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THE MECHANISMS INVOLVED IN THE COLD TOLERANT *TRICHODERMA ATROVIRIDE* BIOCONTROL

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MECHANISMS INVOLVED IN THE COLD TOLERANT *TRICHODERMA ATROVIRIDE* BIOCONTROL

A

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Abstract

Trichoderma atroviride is a cold tolerant fungus that parasitizes a wide range of plant pathogenic fungi. The mechanisms involved in biocontrol by *T. atroviride* are only partially understood. This research evaluated the effect of four different groups of plant pathogenic fungi (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) on enzyme expression at 22 °C and 7 °C. The enzymes expressed (proteinase and endo-β-1,3-glucanase) were purified and characterized, and three 73 kDa N-acetyl-β-D-glucosaminidase genes from three different *T. atroviride* biotypes were sequenced. The β-1,6-glucanase profiles and the regulation of N-acetyl-β-D-glucosaminidases by plant pathogenic fungi were also studied. I document the production of N-acetyl-β-D-glucosaminidase, exochitinase, endochitinase, β-1,3-glucanase, β-1,6-glucanase and proteinase by *T. atroviride* at room temperature. The timing of enzyme expression was pathogen dependent. A high concentration of glucose repressed the expression of glucanases, but had no effect on the expression of N-acetyl-β-D-glucosaminidase. At 7 °C, *T. atroviride* produced the same enzymes as at room temperature except β-1,6-glucanase. The total activities of the chitinases increased over a 30 day incubation period while the expression of glucanases and proteinase depended on the inducer. A new 18.8 kDa serine proteinase and a new 77 kDa endo-β-1,3-glucanase were purified to electrophoretical homogeneity. These two purified enzymes showed strong antifungal activity by inhibiting conidial germination of *Botrytis cinerea*. Three 73 kDa N-acetyl-β-D-glucosaminidase genes were sequenced from *T. atroviride* biotypes 861, 453 and 603. Gene sequences of the enzyme from the *T. atroviride* biotypes are

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different from the published gene sequence of *T. harzianum.* This indicates that the N-acetyl-β-D-glucosaminidase sequence can be used to differentiate the species and isolates of *Trichoderma.* The expression of β-1,6-glucanase is complex and at least three different sizes of β-1,6-glucanase were detected from *T. atroviride.* The expression of β-1,6-glucanase varied with carbon source and pH. Mycelia of plant pathogen regulated the expression of N-acetyl-β-D-glucosaminidase. Two different sizes of N-acetyl-β-D-glucosaminidase were detected when *T. atroviride* was grown with *S. sclerotiorum* and its filtrates. Only one N-acetyl-β-D-glucosaminidase was detected with other pathogens, autoclaved mycelia or glucose. The expression of a 73 kDa N-acetyl-β-D-glucosaminidase was contact-dependent and regulated by an extracellular factor.

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CHAPTER 1: GENERAL INTRODUCTION

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Mechanisms involved in biocontrol by *Trichoderma*

Biological control agents are a potential alternative to chemical fungicides and over 80 biocontrol products have been marketed worldwide. Most biocontrols are used to control plant pathogens in greenhouse systems (30) because plant disease control with microbial agents in commercial agricultural production has been less effective and reliable than with chemical fungicides. Improved biocontrol efficacy requires knowledge of the mechanisms involved in the control of plant pathogens (14, 36). *Trichoderma s*pecies are commonly used to control specific plant diseases. Possible mechanisms involved in disease control by *Trichoderma* include mycoparasitism, antibiosis, competition, and induction of plant defense responses. (14, 15, 20). Mycoparasitism is a primary mechanism reported in biocontrol by *Trichoderma.* Parasitism by *Trichoderma* destroys hypha and causes death of the parasitized fungus. Cell-wall degrading enzymes are induced in *Trichoderma* during the parasitic interaction (4, 28) and a number of *Trichoderma* isolates secrete hydrolytic enzymes such as chitinases, glucanases, lipases, and proteinase when grown in liquid media supplemented with laminarin, chitin, or cell wall constituents of the target fungi (4, 21).

Recent research has focused on elucidating the chitinolytic system of *Trichoderma* because chitin is the main component of fungal cell walls (except the Oomycetes). Most of the *Trichoderma* studies have used *T. harzianum* which produces three types of chitinolytic enzymes (31), i.e. 1,4-β-N-acetylglucosaminidases (EC 3.2.1.30), endochitinase (EC 3.2.1.14) and exochitinases (chitobiosidase or chitin 1,4-chitobiosidase). Two 1,4-β-N-acetyl-D-glucosaminidases, which split the chitin polymer

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into the GlcNAC monomer in an exo-type fashion, are secreted by *T. harzianum* with molecular weights of 102-118 kDa and 73 kDa (15). Four endochitinases which cleave an internal site randomly over the entire length of the chitin microfibril also are expressed by *T. harzianum* with molecular weights of 52 kDa, 42 kDa, 37 kDa and 33 kDa (5, 15, 18). Only one exochitinase (or chitobiosidase) that catalyses the progressive release of diacetylchitobiose in a stepwise fashion is reported from *T. harzianum* (17). Chitinases have cell wall lytic activity as well as antifungal activity (9, 31). CHIT42, CHIT40 and CHIT72 from *T. harzianum* P1 and *T. virens* 41 inhibited spore germination and hyphal elongation of several plant pathogenic fungi (9, 26, 27, 32). Overexpression of a 33-kDa chitinase increased *T. harzianum* antifungal activity against *R. solani* (25). Glucanases are important enzymes produced by *T. harzianum* because glucan is one of the main components of cell walls. There are four types of glucanases involved in biocontrol with *Trichoderma*: exo-1,3-β-glucanase (22), endo-1,3-β-glucanase (7, 28, 34), endo-1,6-β-glucanase (6) and exo-α-1,3-glucanase (1). The 78 kDa β-1,3-glucanase showed direct antifungal activity and was synergistic with chitinase (28) and exo-α-1,3-glucanase which also showed lytic and antifungal activity (1). Endo-β-1,6-glucanase slightly inhibited growth of fungi and was synergistic with endo-β-1,6-glucanase in combination with β-1,3-glucanase, chitinase or both (6).

Fungal proteases may play a significant role in lysis of fungal cell walls (33) because proteins are a component of cell walls of plant pathogenic fungi. Geremia *et al.*, (1993) identified an alkaline proteinase (Prb1) from *T. harzianum* strain IMI 206040 (13) and overproduction of this proteinase improved the biocontrol activity of *T. harzianum* (12).

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The other proteinases also hydrolyzed the cell wall of *Crinipellis perniciosa* (8), inhibited spore germination of *Botrytis cinerea* (3), degraded enzymes produced by *B. cinerea* (11), and served as an elicitor of plant defense compounds (16).

The efficacy of biocontrol by *Trichoderma* in the field is affected by environmental factors (19). Temperature affects *Trichoderma* growth, development, antagonistic activity (19, 23, 37) and efficacy (37). Seeds coated with *T. hamatum* conidia were found to effectively control damping-off caused by *Pythium* or *Rhizoctonia* at temperatures of 17 °C or above but not at 15 °C or below (18). Many economically important plant pathogens, such as *Phytophthora infestans*, *Pythium spp.*, *R. solani* and *S. Sclerotiorum* are mesophiles with strong adaptation to cold temperatures (37). *Trichoderma* isolates adapted t temperature regimes comparable to or better than the plant pathogens they need to control are superior to *Trichoderma* isolates that are incapable of growth or activity throughout the range of temperature (2, 37).

Another factor that affects the success of *Trichoderma* biocontrol is the defense reaction of plant pathogenic fungi. *T. hamatum*, *T. harzianum*, *T. longibrachiatum*, and *T. pseudokoningii* can be parasitized by some forms of plant pathogenic *F. oxysporum* (35). The metabolites produced by plant pathogenic fungi have different effects on biocontrol agents. The metabolites of *R. solani* reduced mycelia growth and conidia production of *T. hamatum*, *T. harzianum*, and *T. viride*; while the metabolites of *P. ultimum* reduced conidia production, but stimulated mycelia, growth of the antagonists (24). Deoxynivalenol (DON) produced by *Fusarium culmorum* and *F. graminearum* repressed the expression of N-acetyl-β-D-glucosaminidase gene, *nag1*, but not the endochitinase

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gene, *ech42* (29). Plant pathogens can defend themselves by detoxification, repressing the genes involved in biocontrol, active efflux of antibiotics, and antibiotic resistance (10). Unfortunately, most information about plant pathogen defense against biocontrol agents is from studying bacterial biocontrol agents and there is very little information about the effects of plant pathogens on biocontrol by *Trichoderma* (10). Understanding fungal pathogen defense can lead to a better understanding of the success or failure of biocontrol.

Trichoderma atroviride strain 901 is a unique fungus found in Alaska that is capable of parasitizing a wide range of plant pathogenic fungi, such as *Rhizoctonia solani*, *Botrytis cinerea*, *Phytophthora cactorum* and *Phytophthora infestans* (37). Although *T. atroviride* has been commercialized, little is known about the molecular mechanisms by which *T. atroviride* is able to parasitize target fungi. Preliminary results showed that *T. atroviride* biotype 901C can produce 1, 4-β-N-acetyl-D-glucosaminidases, endochitinase, exochitinases, β-1.3-glucanase, β-1, 6-glucanase and proteinase when it was grown with autoclaved mycelia of plant pathogens (3). However, which enzymes are produced at a certain time is not known or whether there is a synergistic increase in antifungal activity when different enzymes are combined. Other questions that need to be answered are the effect of temperature and target plant pathogenic fungi on cell wall degrading enzyme production. Answers to these questions are important in understanding the role of these enzymes in *T. atroviride* biocontrol, and in determining the best use of these proteins or the encoding genes in pest management strategies. The goal of this research was to purify the major enzymes produced by *T. atroviride*, to assay antifungal activities of the

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purified enzymes, to determine enzyme production with time, to determine the level of expression of the antifungal protein, to determine the effects of temperature on enzyme production, and to evaluate the effects of target fungi (pathogens) on enzyme production.

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CHAPTER 2: CHITINASE AND GLUCANASE PROFILES OF A COLD TOLERANT *TRICHODERMA ATROVIRIDE* ASSOCIATED WITH PLANT PATHOGENIC PATHOGENS AT ROOM TEMPERATURE

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Abstract

A cold tolerant *Trichoderma atroviride,* biotype 901, produced chitinases and glucanases when the autoclaved mycelia of plant pathogens (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) or glucose were used as carbon sources. The specific enzymes produced were pathogen dependent. The specific activity of N-acetyl-D-glucosaminidase (NAGase), exochitinase, endochitinase, β-1,3-glucanase and β-1,6-glucanase induced by *B. cinerea* was significantly higher than induced by other plant pathogens and glucose. Autoclaved mycelia of *R. solani* were the weakest enzyme inducer. The highest to lowest induction of chitinases (NAGase, Exochitinase and Endochitinase) of *T. atroviride* by autoclaved mycelia was *B. cinerea*, glucose, *P. capsici*, *S. sclerotiorum*, and *R. solani.* The order for induction of β-1,3-glucanase was *B. cinerea*, glucose, *P. capsici*, *R. solani* and *S. sclerotiorum.* The order for β-1,6-glucanase induction was *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* and glucose. Peak enzyme activity occurred after one to six days and was pathogen dependent. The specific activity of NAGase produced by *T. atroviride* was highest no matter what inducing carbohydrate (pathogens, glucose) were used. The specific activity of β-1,6-glucanase was lowest with glucose and autoclaved pathogen mycelia. As a whole, *T. atroviride* produced more chitinases than glucanases. The concentration of glucose also affected enzyme production.

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Introduction

Trichoderma is a common soil inhabiting and fast-growing fungal group found in agriculture, prairie, forest, salt marsh and desert soils (17). This high ecological adaptability, amenability to cultivation and ability to parasitize plant pathogenic fungi, give *Trichoderma* a potential as biocontrol agents (13). The reported mechanisms involved in biocontrol by *Trichoderma spp.* are antibiosis, mycoparasitism, competition and induction of defense responses (9, 10, 11, 13, 14). Mycoparasitism is a major mechanism contributed to *Trichoderma spp.* biocontrol. During mycoparasitism, *Trichoderma* is attracted by a chemical stimulus from plant pathogens, it then recognizes, attaches to and coils the host around the mycelium before cell-wall degrading enzymes destroy the host (3). Cell-wall degrading enzymes associated with *Trichoderma spp.* biocontrol include chitinases, glucanases and proteinases (3). There are three types of chitinolytic enzymes (22), i.e. 1,4-β-N-acetylglucosaminidases (EC 3.2.1.30), endochitinases (EC 3.2.1.14) and exochitinases (or chitobiosidase or chitin 1,4-β-chitobiosidase). The glucanases involved in biocontrol by *Trichoderma* are exo-β-1,3-β-glucanase (16), endo-β-1,3-β-glucanase (6, 18, 26), endo-β-1,6-β-glucanase (5) and exo-α-1,3-glucanase (1).

The kinds of cell-wall degrading enzymes and amount of enzyme produced by *Trichoderma* depend on the strains and carbon sources used (4, 15, 24, 27, 29). Chitin, laminarin, pustulan, and cell walls of plant pathogens induce *Trichoderma* to produce more cell-wall degrading enzymes than other carbon sources (4, 24, 27, 29) and a high concentration of glucose repressed chitinase, chitobiase and 1,3-glucanase (7, 27, 28).

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There is little information comparing enzymes induced in *Trichoderma* by mycelia of plant pathogens with different plant cell wall components (15).

Trichoderma atroviride is a cold tolerant strain and can parasitize a wide range of plant pathogenic fungi (31). Knowledge of the mechanisms of *T. atroviride* biocontrol is limited. For this study, I selected four different plant pathogenic fungi belonging to the Oomycetes (*Phytophthora capsici*), Ascomycetes (*Sclerotinia sclerotiorum*), Basidiomycetes (*Rhizoctonia solani*) and Deuteromycete (*Botrytis cinerea*) to study the relationship between enzyme production and plant pathogenic fungi. I also investigated the effect of glucose concentration on enzyme production.

Materials and Methods

Enzyme induction. *B. cinerea*, *P. capsici*, *R. solani* and *S. sclerotiorum* were selected to evaluate their potential to induce *T. atroviride* cell wall degrading enzymes. Ten day old mycelia of the plant pathogens grown in potato dextrose broth (Difco, Spark, MD) was harvested by filtration on an 0.45 μm Millipore® filter (Bedford, MA), washed three times with sterile water, air dried and ground with mortar and pestle.

T. atroviride biotype 901 conidia harvested from a 10-day old colony were added to 50 ml modified minimal medium broth (30) containing 0.2% asparagine, 0.2% $NaNO_3$, 0.05% $MgSO_4$ $7H_20$, 0.05 % KCl, and 0.1% KH_2PO_4 that was amended with 0.05 % autoclaved mycelia of *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* or glucose to make the final conidial concentration 10^5/ml and incubated in a shaker (150 rpm) at 22 °C. The mycelia of *T. atroviride* were filtered through a 0.45 μm Millipore® filter

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(Bedford, MA) at 0, 1, 2, 3, 4, 5, 6 and 7 days. The protein concentration and enzyme activity in the concentrated filtrate by ultra centrifugal filter devices (Amicon®, Millipore, Bedford, MA) was measured. Each test had three replications and the entire experiment was repeated once.

The effect of glucose on enzyme production. *T. atroviride* conidia were added to 50 ml minimal media broth supplemented with 10 %, 5 %, 2 %, 1 %, 0.5 %, 0.2 %, 0.1 % or 0.05 % glucose and incubated on a shaker (150 rpm) 22 °C for 5 days. Protein was extracted by the procedure described previously. Each treatment had three replications and the whole experiment was repeated once.

Protein assay. Protein concentration was determined using Bradford Reagent (Sigma, St. Louis, MO) (2) when the reaction mixture, containing 2 mL of 30 % Bradford Reagent solution and 50 µL of enzyme preparation was incubated for 30 minutes at room temperature. Protein was measured at 595 nm absorbance compared with a bovine serum albumin standard curve.

Chitinase assay. ρ-Nitrophenyl N-acetyl-β-D-glucosaminide, ρ-Nitrophenyl β-D-N, N'-diacetyl-chitobiose, and ρ-Nitrophenyl β-D-N, N', N''-triacetyl-chitotriose (Sigma, St. Louis, MO) were used as substrates to determine β-N-D-acetylglucosaminidases (EC 3.2.1.30) (NAGase) exochitinase (chitobiosidase, no EC number) and endochitinase (EC 3.2.1.14) activities, respectively (12). One unit activity was defined as the release of 1 µmol nitrophenol from the substrate per minute (12). The reaction mixture contained 30 µl of enzyme solution and 50 µl of 100 µg/ml substrate, was incubated at 50 °C for 15

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minutes before adding 50 µl of 0.4 M sodium carbonate and measuring absorbance at 410 nm.

Glucanase assay. β-1, 3-endoglucanase, β-1, 4-endoglucanase and β-1, 6-endoglucanase activities were detected using laminarin (Sigma, St. Louis, MO), carboxymethyl cellulose (CMC) (Sigma, St. Louis, MO) and pustulan (Calbiochem, La Jolla, CA) as substrates, respectively (21, 25). The reducing-sugar content was determined with a procedure modified from Nelson (25) where one unit of endoglucanase activity corresponds to the release of 1 µmol of glucose per minute. The reaction mixture containing 80 µl of enzyme solution, 160 µl of 50 mM potassium acetate buffer (pH 5.0) and 160 µl of 0.5 % substrate in 50 mM potassium acetate buffer (pH 5.0) was incubated at 37 °C for 30 minutes. Then 1 ml of solution A (Solution A Contained 40.0 g/L Na_2CO_3, 16.0 g/L glycine and 0.45 g/L $CuSO_4$) and 1 ml of solution B (Solution B contained 0.12 g/L neocuproine hydrochloride) were added to the mixture, boiled for 10 minutes and the absorbance measured at 440 nm.

Statistical analysis. All experiments were designed with three replications and repeated once. The data were analysed using ANOVA and the GLM procedure of SAS with lsmeans (SAS Institute Inc. 1999) (23).

Results

The effect of pathogen mycelia on the production of cell wall degrading enzymes. *T. atroviride* biotype 901 produced N-acetyl-β-D-glucosaminidase (NAGase), exochitinase, endochitinase, β-1,3-glucanase, and β-1,6-glucanase when it was grown in minimal

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media supplemented with autoclaved mycelia of plant pathogenic pathogens or glucose (Figures 2.1-2.5). The amount of enzyme produced and timing of production were pathogen dependent. The specific activities of all five tested enzymes were the highest when *T. atroviride* was grown in the minimal media supplemented with autoclaved mycelia of *B. cinerea* (Figures 2.1-2.5). *R. solani* mycelia promoted the least cell wall degrading enzyme induction. Specific activities of three chitinases were lowest when *T. atroviride* was grown in minimal media supplemented with autoclaved mycelia of *S. sclerotiorum* (Figures 2.1-2.5); even lower than glucose as the sole carbon source. *T. atroviride* produced NAGase regardless of the mycelium or glucose source used.

Time-course of NAGase production with different inducers. The specific activity of NAGase produced by *T. atroviride* was the highest when *T. atroviride* was grown in minimal media supplemented with autoclaved mycelia of *B. cinerea*. The order for production of NAGase was *B. cinerea*, glucose, *P. capsici*, *S. sclerotiorum* and *R. solani*. The specific activity of NAGase peaked six days, two days, three days, and the first day for mycelia of *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* and glucose, respectively (Figure 2.1).

Time-course of exochitinase production with different inducers. Autoclaved mycelia of *B. cinerea* also induced *T. atroviride* to produce the most exochitinase (Figure 2.2). The order for inducing exochitinase was *B. cinerea* mycelium, glucose, *P. capsici* mycelium, *S. sclerotiorum* mycelium, and *R. solani* mycelium. The specific activity of exochitinase peaked at the day five, day two, day five, day one and day one for mycelia of *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* and glucose, respectively (Figure 2.2).

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Time-course of endochitinase production with different inducers. The induction of endochitinase followed the same pattern as the induction of NAGase and exochitinase. Autoclaved mycelia of *B. cinerea* induced the most endochitinase. The order for induction was *B. cinerea*, glucose, *P. capsici*, *S. sclerotiorum* and *R. solani* (Figure 2.3).

The effect of plant pathogens and glucose on glucanase production. Specific activities of β-1,3-glucanase and β-1,6-glucanase were lower than the specific activity of chitinases (Figure 2.4, Figure 2.5). *B. cinerea* was still the strongest inducer of β-1,3-glucanase and β-1,6-glucanase. The order of specific activity of β-1,3-glucanase were *B. cinerea*, glucose, *P. capsici*, *R. solani*, and *S. sclerotiorum* (Figure 2.4), while the order of specific activity of β-1,6-glucanase were *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* and glucose (Figure 2.5).

The effect of glucose concentration on enzyme production. High concentrations of glucose inhibited β-1,3-glucanase and β-1,6-glucanase production. There was no β-1,3-glucanase or β-1,6-glucanase detected at 10 % and 5 % glucose. The highest specific activities of β-1,3-glucanase and β-1,6-glucanase were detected when 0.1 % glucose was added to the media. Glucose had no effect on NAGase activity. Very low exochitinase and endochitinase activities were detected at higher glucose concentrations (Table 2.1).

Discussion

Trichoderma species produce various cell wall degrading enzymes (4, 15, 24, 27, 28, 29). When not easily metabolized carbon sources, such as chitin, chitin degradation products, laminarin, pustulan, nigeran, cell walls of *R. solani* and *B. cinerea* were added to media,

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higher specific activities of cell wall degrading enzymes were detected (4, 24, 27, 28, 29). Autoclaved mycelia of *B. cinerea* induced more cell wall degrading enzymes than glucose, but the addition mycelia of *P. capsici*, *R. solani* and *S. sclerotiorum* generally resulted in lower cell wall degrading enzyme production than glucose (except β-1,6-glucanase when *T. atroviride* was grown in the media supplemented with autoclaved mycelia) (Figures 2.1-2.5). The time course for cell wall degrading enzyme production by *T. atroviride* was also pathogen dependent. The cell wall degrading enzymes induced by autoclaved mycelia of *B. cinerea* peaked 4-6 days and the enzymes induced by other plant pathogens peaked during the first three days. This may indicate that different cell wall and cytoplasmic components become available in a sequential manner. Autoclaved mycelia are less complex than those of cell walls of living plant pathogens because living cells contain not only cell wall components but also cytoplasmic components. This could provide more nutritive and supplemental factors than cell walls used as the sole carbon source. In nature, cell wall degrading enzymes produced by *Trichoderma* are affected not only by components of cell walls but also by cytoplasmic and metabolic components of plant pathogens. Using autoclaved mycelia of plant pathogenic fungi as a carbon source provides a simpler way to determine the responses of *Trichoderma* with its host.

The cell wall degrading enzymes produced by *Trichoderma* were repressed when a high level of glucose was added to media (7, 27, 28); however, cell wall degrading enzymes could be detected when glucose was used as the sole carbon source (27, 28). *T. atroviride* also produced cell wall degrading enzymes when glucose was used as the sole carbon source. Specific activity of cell wall degrading enzymes produced by *T. atroviride* were

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higher when a low concentration of glucose (0.05 %) was used as the sole carbon source than when autoclaved mycelia of *P. capsici*, *R. solani*, and *S. sclerotiorum* were used (Figures 2.1-2.5). This could be an effect of degrading products in autoclaved mycelia of plant pathogens other than in cell walls of living fungal pathogens. Some cytoplasmic components could affect the cell wall degrading enzyme production because regulation of cell wall degrading enzymes produced by *Trichoderma* is more complex with mycelia than glucose. Another reason for this may be the involvement of higher nitrogen repression (7). In the media, the total nitrogen sources are about 40 mM. The total nitrogen sources increased when the autoclaved mycelia of plant pathogens added into the media and the concentration may be near 100 mM when the autoclaved mycelia of *P. capsici*, *R. solani* and *S. sclerotiorum* were used as inducer and caused the repression of the expression of cell wall degrading enzymes. *T. harzianum* produced β-1,3-glucanase, β-1,6-glucanase, and endochitinase were inhibited by a high concentration of glucose (10 %) which NAGase was less affected by a concentration (4). Enzymes of *T. atroviride* were similarly affected. At the higher concentrations of glucose (5-10 %), no β-1,3-glucanase and β-1,6-glucanase were detected (Table 2.1); however glucose had no effect on NAGase production. In contrast to *T. harzianum*, exochitinase and endochitinase of *T. atroviride* could be detected only when the glucose concentration were higher. Interactions between *Trichoderma* and plant pathogens involve more than just the enzymes than described here. Most studies of interactions between *Trichoderma* and plant pathogens have focused on responses of *Trichoderma* to the pathogens with less attention focused on the plant pathogen defense responses (8). Deoxynivalenol (DON)

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produced by *Fusarium culmorum, F. graminearum* repressed the expression of NAGase (*nag1*) expression (20). Some metabolites from the autoclaved mycelia of *P. capsici, R. solani* and *S. sclerotiorum* may play the same role as DON and repressed the expression of cell wall degrading enzymes from *T. atroviride* because the specific activities of cell wall degrading enzymes were lower than glucose. Studies on interactions *in vivo* and *in situ* between *Trichoderma* and fungal plant pathogens or plants may provide a better understanding of *Trichoderma* biocontrol mechanisms (19).

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Table 2.1. The effect of glucose concentration on enzyme production.

	Specific Activity				
Glucose concentration	β-1,3-glucanase	β-1,6-glucanase	NAGase	Exochitinase	endochitinase
10 %	0^{b}	0.208^{b}	61.73^{a}	14.43^{a}	0.86^{b}
5 %	0^{b}	0^{b}	124.89^{a}	8.91^{abc}	8.2^{a}
2 %	2.10^{b}	0.88^{b}	160.41^{a}	11.29^{ab}	0^{b}
1 %	7.20^{b}	1.82^{b}	102.33^{a}	3.66^{bc}	0^{b}
0.5 %	25.66^{ab}	5.22^{b}	36.92^{a}	0^{c}	0^{b}
0.2 %	35.05^{ab}	8.02^{ab}	176.12^{a}	0^{c}	0^{b}
0.1 %	81.13^{a}	16.21^{a}	192.11^{a}	1.69^{bc}	0^{b}
0.05 %	8.13^{b}	0.87^{b}	169.61^{a}	0^{c}	0^{b}

*: The unit of specific activity was mU/mg protein; the average of six replications from two experiments; values followed by the same letter are not significantly different according to t-test ($LSD_{0.05}$). NAGase=N-acetyl-β-D-glucosaminidase.

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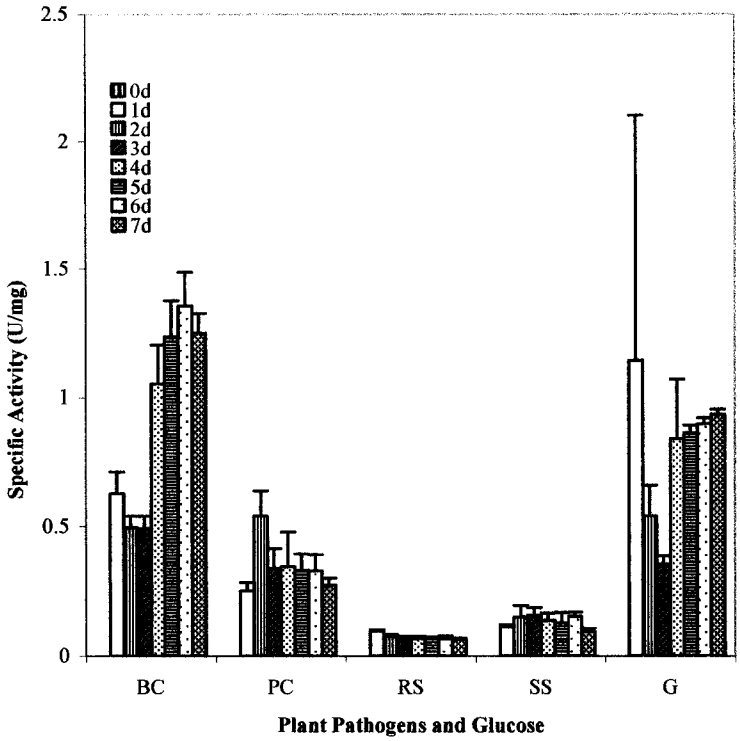

Figure 2.1. Specific activity of N-acetyl-glucosaminidase (NAGase). The NAGase produced by *Trichoderma atroviride* biotype 901 provided 0.05% autoclaved mycelium of different plant pathogens. Values are the average of three replications. The error bars represent the standard errors. BC=autoclaved mycelia of *Botrytis cinerea*; PC=autoclaved mycelia of *Phytophthora capsici*; RS=autoclaved mycelia of *Rhizoctonia solani*; SS=autoclaved mycelia of *Sclerotinia sclerotiorum*; G=glucose; d=days.

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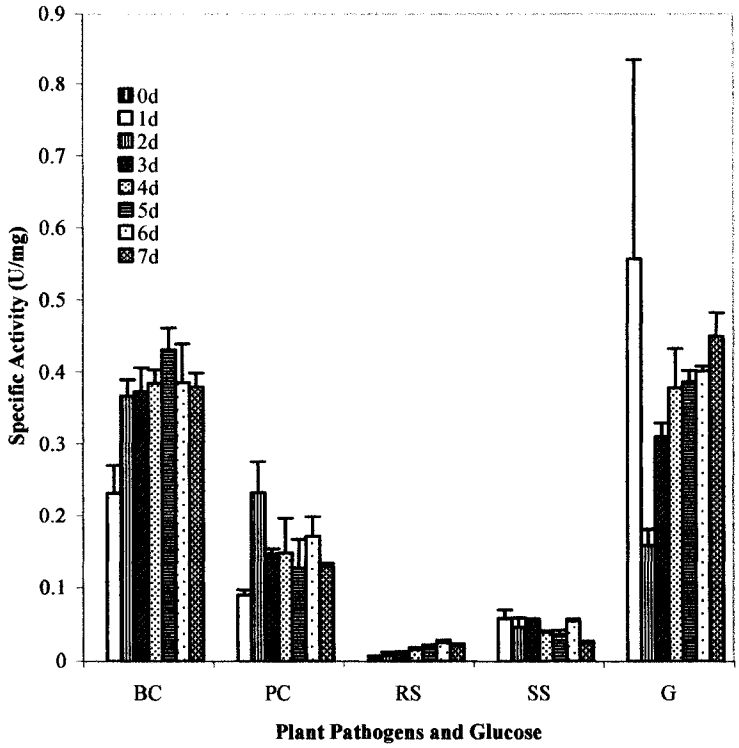

Figure 2.2. Specific activity of exochitinase. The enzyme produced by *Trichoderma atroviride* biotype 901 provided 0.05% autoclaved mycelia of plant pathogens with time. Specific activities in the two experiments were similar. Values shown are the average of three replications. The error bars represent the standard errors. BC=autoclaved mycelia of *Botrytis cinerea*; PC=autoclaved mycelia of *Phytophthora capsici*; RS=autoclaved mycelia of *Rhizoctonia solani*; SS=autoclaved mycelia of *Sclerotinia sclerotiorum*; G=glucose; d=days.

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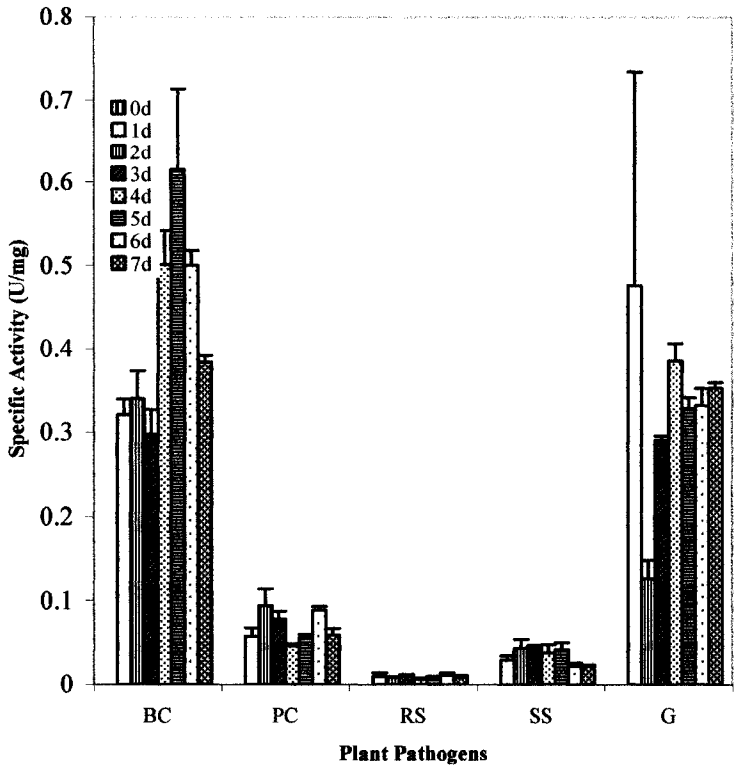

Figure 2.3. Specific activity of endochitinase. The enzyme produced by *Trichoderma atroviride* biotype 901 provided 0.05% autoclaved mycelia of plant pathogens with time. Value is the average of three replications. The error bars represent the standard errors. BC=autoclaved mycelia of *Botrytis cinerea*; PC=autoclaved mycelia of *Phytophthora capsici*; RS=autoclaved mycelia of *Rhizoctonia solani*; SS=autoclaved mycelia of *Sclerotinia sclerotiorum*; G=glucose d=days.

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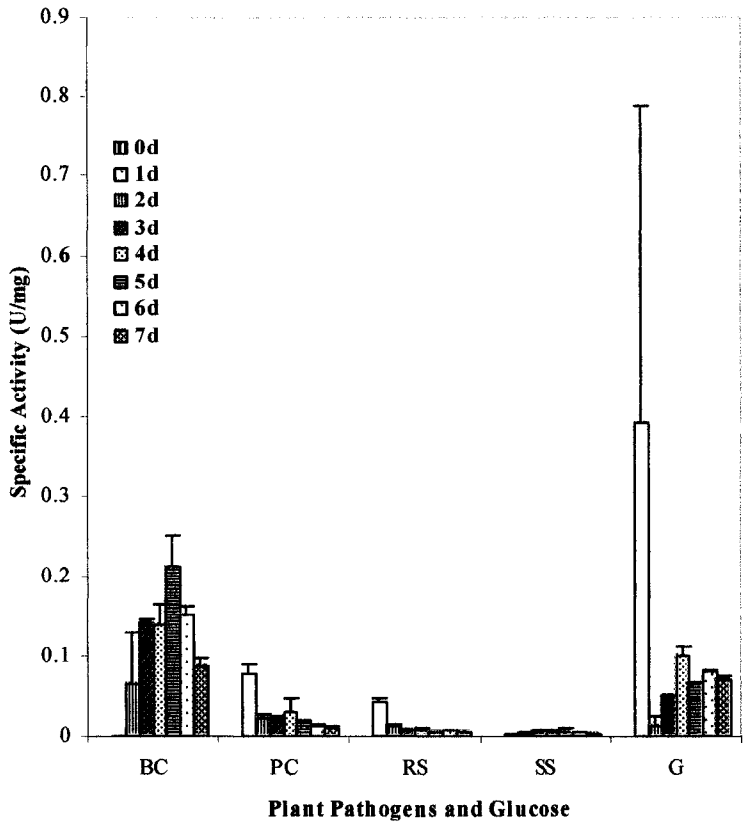

Figure 2.4. Specific activity of β-1,3-glucanase. The enzyme produced by *Trichoderma atroviride* biotype 901 provided 0.05 % autoclaved mycelia of plant pathogens with time. Values are the average of three replications. The error bars represent the standard errors. BC=autoclaved mycelia of *Botrytis cinerea*; PC=autoclaved mycelia of *Phytophthora capsici*; RS=autoclaved mycelia of *Rhizoctonia solani*; SS=autoclaved mycelia of *Sclerotinia sclerotiorum*; G=glucose; d=days.

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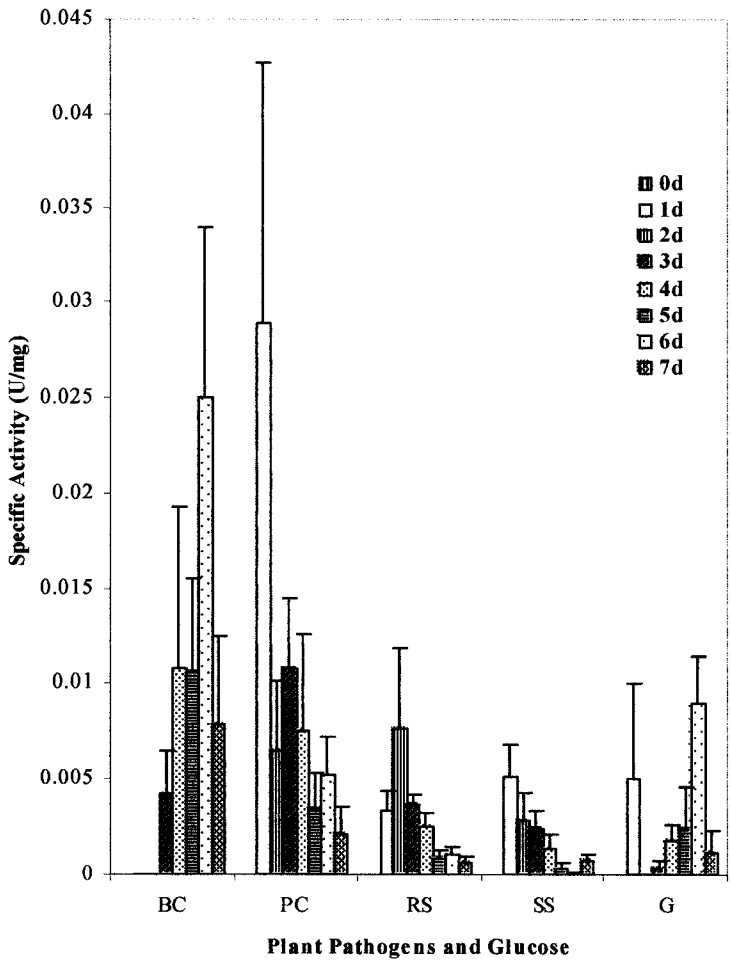

Figure 2.5. Specific activity of β-1,6-glucanase. The enzyme produced by *Trichoderma atroviride* biotype 901 with 0.05 % autoclaved mycelia plant pathogens with time. Values shown are the average of three replications. The error bars represent the standard errors. BC=autoclaved mycelia of *Botrytis cinerea*; PC=autoclaved mycelia of *Phytophthora capsici*; RS=autoclaved mycelia of *Rhizoctonia solani*; SS=autoclaved mycelia of *Sclerotinia sclerotiorum*; G=glucose; d=days.

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CHAPTER 3: ENZYME PROFILES OF A COLD TOLERANT *TRICHODERMA ATROVIRIDE* IMPLICATED IN LOW TEMPERATURE BIOCONTROL OF PLANT PATHOGENIC FUNGI

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Abstract

Trichoderma atroviride, a cold tolerant biocontrol agent that parasitizes a wide range of plant pathogenic fungi, produces cell-wall degrading enzymes at low temperatures in the presence to plant pathogenic fungi. *T. atroviride* 901 was grown at 7 °C in minimal medium broth amended with or without autoclaved mycelia of *Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Appropriate substrates and enzyme standards were used to detect the presence of cell wall degrading enzymes. *T. atroviride* β-1,4-N-D-acetylglucosaminidase (NAGase), exochitinase, endochitinase, β-1,3-endoglucanase and proteinase activity were all increased in the presence of fungal pathogens compared to activity in the absence of pathogens. The total activity of all chitinases increased over a 30-day period. Increased specific activity of NAGase, exochitinase and endochitinase were detected 5 days after inoculation. The timing of β-1,3-endoglucanase and proteinase production was pathogen dependent. β-1,3-glucanase activity in the presence of autoclaved mycelia of *S. sclerotiorum*, *P. capsici*, *R. solani*, *B. cinerea* and glucose was detected at 5, 10, 15, 20, and 25 days, respectively. Specific proteinase activity peaked at 10 days and 20 days for *S. sclerotiorum* and *P. capsici*, respectively. No detectable proteinase activity was observed when the autoclaved mycelia of *R. solani*, *B. cinerea* and glucose were used as inducers.

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Introduction

Trichoderma species have been used to control plant diseases in many crops (5). However, the success of the *Trichoderma* application is greatly influenced by environmental factors (5). Temperature is one of the environmental factors affecting *Trichoderma* growth, development and efficacy for disease control (5,7,11,16). Seeds coated with *T. hamatum* conidia effectively controlled damping-off caused by *Pythium* or *Rhizoctonia* at temperatures of 17 °C and higher, but not at 15 °C or lower (3). Many economically important plant pathogens such as *Phytophthora infestans*, *Pythium spp.*, *R. solani* and *Sclerotinia sclerotiorum* are mesophiles with strong adaptation to cold temperatures (16). *Trichoderma* species adapted to comparable or lower temperatures than plant pathogens provide superior disease control compared to *Trichoderma* isolates that are incapable of growth or activity at cold temperatures (1,16). Information on biocontrol by *Trichoderma* at low temperatures is limited (1,6,8,9,10,14,16). In this chapter, I report the enzyme profiles produced by a cold tolerant *T. atroviride* (biotype 901) at 7 °C to identify several biocontrol mechanisms that can function at low temperatures.

Materials and Methods

Preparation of plant pathogens. *Botrytis cinerea*, *P. capsici*, *R. solani* and S. *sclerotiorum* were selected as pathogens for control. These pathogens were grown in potato dextrose broth (Difco, Spark, MD) for 10 days at room temperature. The mycelia were collected

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by vacuum filtration on 0.45 μm Millipore® filter paper (Bedford, MA), washed three times with sterile water, dried and ground.

Enzyme induction. *T. atroviride* biotype 901 conidia harvested from a 10-day old PDA agar culture were added to 50 ml modified minimal medium broth containing 0.2% asparagine, 0.2% $NaNO_3$, 0.05% $MgSO_4$ $7H_20$, 0.05 % KCl, 0.1% KH_2PO_4 (15) amended with 0.05 % autoclaved mycelia of *B. cinerea*, *P. capsici*, *R. solani*, *S. sclerotiorum* or glucose to make the final conidia concentration 10^5ml^{-1} and incubated in a shaker (150 rpm) at 7 °C for 0, 5, 10, 15, 20, 25 and 30 days. The mycelia of *T. atroviride* were filtered through 0.45 μm Millipore® filter paper (Bedford, MA) and the filtrate was concentrated by Amicon® Ultra centrifugal filtration (Millipore, Bedford, MA). The protein concentration and enzyme activity were measured at each harvest period. Each test had three replications and the entire experiment was repeated twice.

Determination of protein concentration. Protein concentration was determined using Bradford Reagent (Sigma, St. Louis, MO). The reaction mixture containing 2 mL of 30 % Bradford Reagent solution and 50 μL of enzyme preparation was incubated for 30 minutes at room temperature. The protein concentration of the sample was determined at 595 nm absorbance according to the standard curve using bovine serum albumen as a standard.

Chitinase assays. ρ-Nitrophenyl N-acetyl-β-D-glucosaminide, ρ-Nitrophenyl β-D-N, N'-diacetyl-chitobiose, and ρ-Nitrophenyl β-D-N, N',N''-triacetyl-chitotriose (Sigma, St. Louis, MO) were used as substrates to determine β-N-D-acetylglucosaminidases (EC 3.2.1.30) (NAGase), chitobiosidases (exochitinase, no EC number) and endochitinase

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(EC 3.2.1.14) activity, respectively (4). One unit activity was defined as the release of 1 μmol nitrophenol from substrate per minute (4). The reaction mixture, containing 30 μl of enzyme solution and 50 μl of 100 μg/ml substrate, was incubated at 50 °C for 15 minutes before adding 50 μl of 0.4 M sodium carbonate. Absorbance was measured at 410 nm.

Glucanases assays. β-1,3-endoglucanase, β-1,4-endoglucanase and β-1, 6-endoglucanase activities were detected by using laminarin (Sigma, St. Louis, MO), carboxymethyl cellulose (CMC) (Sigma, St. Louis, MO) and pustulan (Calbiochem, La Jolla, CA) as substrates, respectively (12, 13). The reducing-sugar content was determined with a procedure modified from Nelson (12) where one unit of endoglucanase activity corresponds to the release of 1 μmol of glucose in one minute. The reaction mixture, containing 80 μl of enzyme solution, 160 μl of 50 mM potassium acetate buffer,(pH 5.0), and 160 μl of 0.5 % substrate in 50 mM potassium acetate buffer (pH 5.0), was incubated at 37 °C for 30 minutes. Then, 1 ml of solution A (Solution A Contained 40.0 g/L Na_2CO_3, 16.0 g/L glycine and 0.45 g/L $CuSO_4$) and 1 ml of solution B (Solution B contained 0.12 g/L neocuproine hydrochloride) were added to the mixture, boiled for 10 minutes and measured at 440 nm.

Proteinase assay. The proteinase assay followed the procedure described by Beynon and Bond (2). One unit of proteinase activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette per min at 440 nm (2). The reaction mixture contained 50 μl of enzyme preparation and 100 μl of 2 % azocasein (Sigma, St. Louis, MO) in 50 mM pH 7.0 potassium phosphate buffer. The mixture was

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incubated at 25 °C for 30 minutes before adding 1.2 mL of 5 % trichloroacetic acid (TCA) (VWR, Bristol, CT), centrifuging at 12,000 rpm for 5 minutes, Mixing 1.2 ml of supernatant with 0.8 ml of 1 M sodium hydroxide and assaying at 440 nm.

Results

T. atroviride biotype 901 produced higher levels of β-1,4-N-D-acetylglucosaminidase (NAGase), exochitinase, endochitinase, β-1,3-glucanase and proteinase at 7 °C when it was grown in minimal media broth supplemented with autoclaved mycelia of *B. cinerea*; *P. capsici*; *R. solani* or *S. sclerotiorum* than with glucose (Figures 3.1, 3.2, 3.3 and Tables 3.1 and 3.2). The total activity of NAGase, exochitinase and endochitinase continued to increase throughout the 30-day test period both with and without mycelia. (Figures 3.1, 3.2 and 3.3). The only exception was the peaking of exochitinase activity at 15 days when *T. atroviride* was grown with autoclaved *R. solani* mycelia (Figure 3.2). Production of β-1,3-glucanase was pathogen dependent and detected after 5, 10, 15, 20 and 25 days incubation in the presence of *S. sclerotiorum*, *P. capsici*, *R. solani*, *B. cinerea* and glucose, respectively (Table 3.1). Proteinase was detected after 5 days and 10 days, and peaked at 20 days and 10 days, when *T. atroviride* was grown with autoclaved mycelia of *P. capsici* and *S. sclerotiorum*, respectively (Table 3.2). No proteinase was detected when *T. atroviride* was grown in the presence of autoclaved *B. cinerea* or *R. solani* mycelia or glucose (Table 3.2). β-1,4-glucanase and β-1,6-glucanase activity were not detected in any of the tests.

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Discussion

Temperature is a determining factor for efficacy of biological control of plant diseases. Higher destructive plant pathogens such as *Phytophthora infestans*, *R. solani*, and *S. sclerotiorum* can grow at 5 °C, 1 °C and 4 °C, respectively (16). To control these diseases effectively, biocontrol agents must have temperature adaptability equal to or better than the targeted plant pathogen. The results show that *T. atroviride* produces cell wall degrading enzymes in response to specific plant pathogen mycelia at 7 °C and indicate the mechanism for its reported biocontrol activity at low temperature (16).

Chitin and glucan are the two major components of fungal cell walls. This research shows that autoclaved mycelia of *B. cinerea, P. capsici, R. solani* and *S. sclerotiorum* induce chitinase (NAGase, exochitinase and endochitinase) and β-1,3-glucanase production. Specific enzyme production of *T. atroviride* was highly dependant on specific pathogens. For instance, β -1,3-glucanase production in *T. atroviride* was induced by *S. sclerotiorum* in 5 days while it took 20 days *with B. cinerea*. Although chitinases, e.g. N-acetyl-D-glucosaminidase, exochitinase, and endochitinase are produced by *T. atroviride* routinely, their production increases dramatically in the presence of mycelia of all plant pathogens tested.

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Table 3.1. β-1,3-glucanase activity of *T. atroviride* with pathogen mycelia and glucose.

Time	The autoclaved mycelia of pathogens				
(Days)	*B. cinerea*	*P. capsici*	*R. solani*	*S. sclerotiorum*	glucose
0	ND[a]	ND	ND	ND	ND
5	ND	ND	ND	6.25	ND
10	ND	14.5	ND	8	ND
15	ND	122	98.4	429	ND
20	14.1[b]	935	64.4	580	ND
25	26.3	625	64.3	259	55.6
30	31.1	250	156.5	216	56.7

[a]: not detectable.[b]: Total activity (mU).

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Table 3.2. Proteinase activity of *T. atroviride* with pathogen mycelia and glucose.

Time	The total activity (mU)		The specific activity (U/mg)	
(Days)	*P. capsici*	*S. sclerotiorum*	*P. capsici*	*S. sclerotiorum*
0	ND[a]	ND	ND	ND
5	3.82	ND	2.28	ND
10	5.46	4.11	1.96	0.85
15	12.57	4.55	1.56	0.39
20	25.64	6.12	5.27	0.18
25	9.16	6.98	0.25	0.22
30	6.37	8.51	0.09	0.21

[a]: not detectable.

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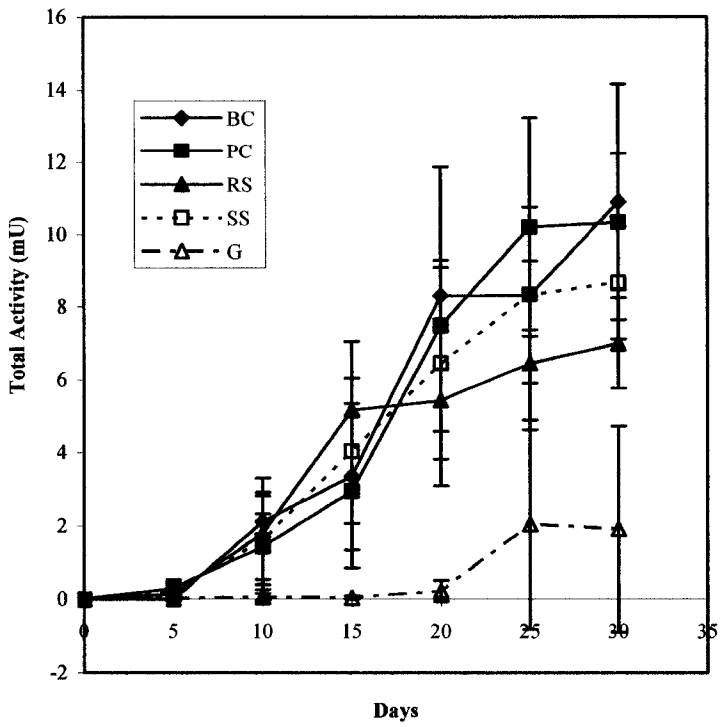

Figure 3.1. Total N-acetyl-D-glucosaminidase activity. N-acetyl-D-glucosaminidase (NAGase) produced by *T. atroviride* 901 with mycelia of plant pathogenic fungi or glucose at 0, 5, 10, 15, 20, 25 and 30 days. The value based on the combined average of three replications and two experiments. The error bars indicate standard deviations.

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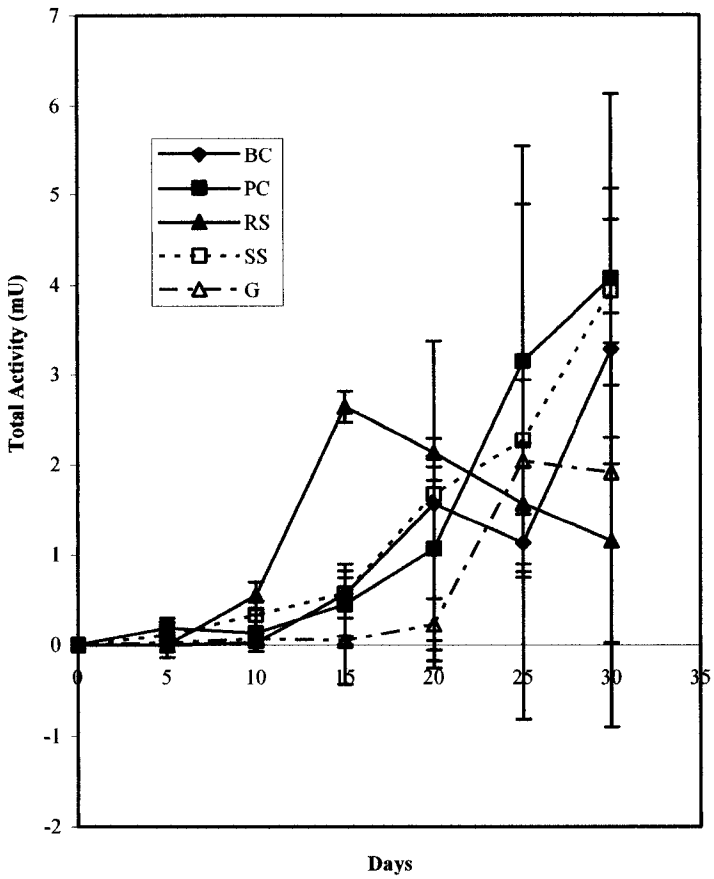

Figure 3.2. Total exochitinase activity. The exochitinase produced by *T. atroviride* 901 with mycelia of plant pathogenic fungi and glucose at 0, 5, 10, 15, 20, 25 and 30 days. Each test had three replications and repeated twice. The graph showed the combined results of two experiments. The error bars indicated standard deviations.

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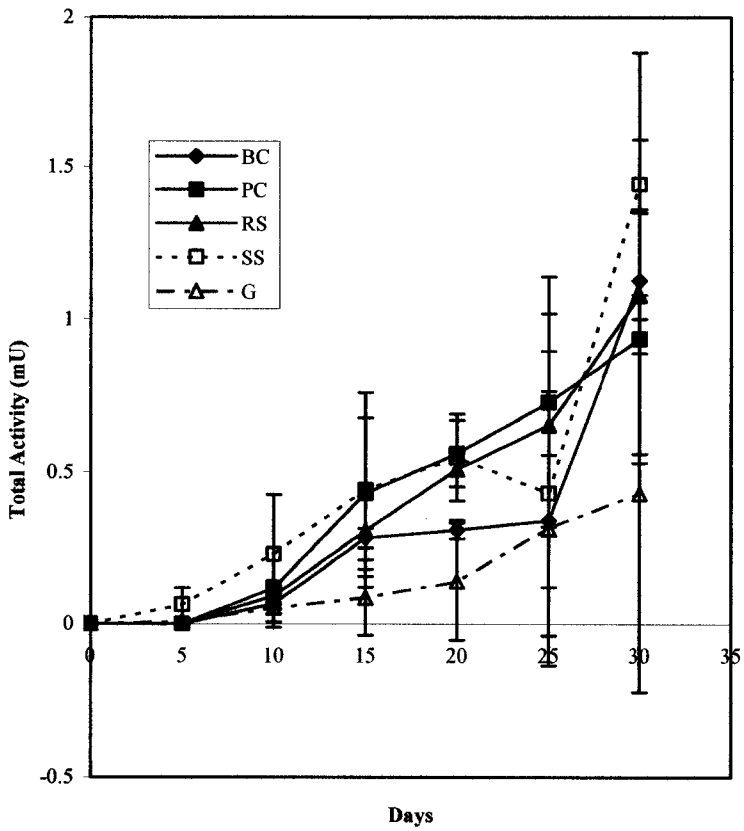

Figure 3.3. Total endochitinase activity. The Endochitinase produced by *T. atroviride* 901 with mycelia of plant pathogenic fungi or glucose at 0, 5, 10, 15, 20, 25 and 30 days incubation. Each test had three replications and repeated twice. The graph showed the combined results of two experiments. The error bars indicated standard deviations.

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CHAPTER 4: CHARACTERIZATION OF A SERINE PROTEINASE PRODUCED BY *TRICHODERMA ATROVIRIDE*

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Abstract

A cold tolerant *Trichoderma atroviride* strain isolated from Alaska soils has strong biocontrol potential against diverse plant pathogens; however, the mechanisms involved in mycoparasitism have not been completely elucidated. Specific *Trichoderma* proteinases may play a role in lysis of fungal plant pathogens by degrading fungal cell walls. To study the role of proteinase in mycoparasitism, autoclaved mycelia of *R. solani*, *S. sclerotiorum, B. cinerea* and *P. capsici* were used to induce proteinase in *T. atroviride*. The time for specific induction of proteinase activity was dependent on the pathogen and ranged between one to three days. One proteinase purified to electrophoretical homogeneity has a molecular weight of 18.8 kDa and an isoelectric point of 8.5. Phenylmethylsulfonyl fluoride (PMSF) can totally inhibit the activity of this proteinase and implicates it as a serine proteinase. The optimal pH of this proteinase was 9.0. The purified proteinase inhibited conidial germination of *B. cinerea* by 80-86.7 %. Amino terminal amino acid sequencing of the proteinase revealed an amino acid sequence of IVGGTTAAAG. A database homology search using the BLASTp algorithm showed greatest similarity to an extracellular protease from *Cochliobolus carbonum*.

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Introduction

Trichoderma species are used to control plant pathogenic fungi (20, 22). The mechanisms involved in biocontrol include the production of antibiotics, competition, and mycoparasitism induction of plant defense responses (19, 22, 23). Mycoparasitism is an important mechanism in *Trichoderma* biocontrol (21). Cell-wall-degrading enzymes are induced in *Trichoderma* during the parasitic interaction (2, 4, 6, 8, 11, 16). These lytic enzymes include chitinases (2, 12, 22, 24, 25, 26, 27), β-glucanases (6, 7, 8, 14, 17, 27, 32, 34, 35, 39) and proteinases (13, 14, 15, 16, 19, 29). Chitinases and glucanases have direct antifungal activity (6, 7, 8, 12). Since proteins are components of the cell-wall skeleton of plant pathogens (5), proteinases may play a role in mycoparasitism by degrading fungal cell walls. Culture filtrates containing proteinase can inhibit conidial germination of *B. cinerea* and deactivate the hydrolytic enzymes produced by *B. cinerea* (13). There are several reports on the purification and function of proteinase (10, 15, 16, 18, 19). Overproduction of a 31 kDa proteinase improved the biocontrol activity of *Trichoderma* (16). Other proteinases include an 18.8 kDa proteinase from *T. harzianum* which hydrolyzed the cell wall of *Crinipellis perniciosa* (11); an 18 kDa serine proteinase from *T. virens* that stimulated cotton terpenoid production, increased peroxidase activity and served as an elicitor of plant defense compounds (19); and a 36.7 kDa aspartyl protease from *T. harzianum* CEC2413 (10).

Trichoderma atroviride 901 derived from a cold tolerant strain isolated from Alaskan soils can parasite a wide range of plant pathogens including *Rhizoctonia solani*, *Botrytis cinerea*, *Phytophthora cactorum* and *Phytophthora infestans* (40). It also produces

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extracellular enzymes such as chitinases, β-glucanases and proteinase (3). To better understand the mechanisms involved in biocontrol by plant pathogens of *T. atroviride*, several enzymes implicated in references biocontrol have been isolated from *T. atroviride* and tested for antifungal activity (3,6,7,24,25,28). In this chapter, I report the purification and characterization of a serine proteinase from *T. atroviride*. The probable role of this enzyme in mycoparasitism by *T. atroviride* also is discussed.

Materials and Methods

Reagents. Azocasein, phenylmethylsulfonyl fluoride (PMSF), N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutyamide (E64), pepstatin A, and 1,10-phepathroline, DEAE ion exchanger were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol (PEG), 12% precast iGel, 1.5 mL disposable semi-micro cuvettes, and trichloroacetic acid (TCA) were purchased from VWR (Bristol, CT), and Bio-Rad gel p-60, hydroxylapatite, SDS-PAGE low range standard and IEF standard were purchased from Bio-Rad (Hercules, CA). Carboxymethyl (CM) Cellulose cation exchanger was purchased from Pharmacia LKB (Uppsala, Sweden).

Growth conditions. *T. atroviride* conidia harvested from a 10-day old potato dextrose agar colony were introduced into one liter of modified minimal medium (0.2% asparagine, 0.2% $NaNO_3$, 0.05% $MgSO_4$ $7H_20$, 0.05 % KCl, 0.1% KH_2PO_4 and 0.05% skimmed milk powder) (38) to make the final conidia concentration 10^5/ml and incubated on a rotary shaker for three days at 150 rpm. Mycelium of *T. atroviride* was removed by vacuum filtration (VWR, Bristol, CT) and proteins in the filtrate were concentrated by

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polyethylene glycol 20,000 (PEG).

Proteinase assay. The proteinase assay followed the procedure described by Beynon et al (1) where, one hundred and fifty µl mixture containing 50 µl of enzyme sample and 100 µl of 2 % azocasein (Sigma, A-2765, St. Louis, MO) were incubated at 25 °C for 30 minutes before adding 1.2 mL of 5 % trichloroacetic acid (TCA) and centrifuging at 12,000 rpm for 5 minutes. The supernatant (1.2 mL) and 0.8 mL of 1 M sodium hydroxide were mixed in a cuvette and the absorbance was measured at 440 nm. One unit of proteinase activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette per minute under conditions of the assay.

Determination of protein. Protein concentration of the filtrate was determined using Bradford Reagent (Sigma, St. Louis, MO) from a mixture containing 2 mL of 30 % Bradford Reagent solution and 50 µL of the enzyme sample incubating for 30 minutes at room temperature, and measuring protein in the sample by it's absorbance at 595 nm. BSA was used as a standard.

Purification of proteinase. The fungal filtrate was transferred into dialysis tubing (6,000-8,000 Da cut-off) and concentrated by placing the dialysis tubing in solid polyethylene glycol (PEG) (20,000 Mr) as described by Di Pietro et al. (12). The concentrate from PEG was subsequently dialyzed overnight against 50 mM potassium phosphate buffer, at pH 7.0 and applied to a DEAE anion exchange chromatography column (1 by 20 cm). The column was eluted with the same buffer at 1ml per minute and sequential 5 ml fractions were collected. Fractions with proteinase activity from DEAE chromatography were pooled together and applied to a carboxymethyl (CM) cellulose cation exchange

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chromatography column (1 by 20 cm) directly. The elution buffer was 50 mM potassium phosphate buffer, pH 7.0. The elution rate was 1mL/min and 5 ml sequential fractions were collected. Fractions with proteinase activity from carboxymethyl (CM) cellulose chromatography were pooled together, concentrated with PEG, dialyzed against 20 mM HEPES buffer (Sigma, St. Louis, MO), pH 7.0, and applied to a Hydroxylapatite (Bio-Gel® HTP from Bio-Rad) column (1 by 20 cm) equilibrated with 20 mM HEPES buffer, pH 7.0 (32). The column was eluted with 30 mL of 20 mM pH 7.0 HEPES buffer, then 20 mL of 20 mM pH 7.0 HEPES buffer with 10 mM sodium phosphate, then 20 mL of HEPES with 25 mM sodium phosphate and then 40 mL of HEPES with 100 mM sodium phosphate. The flow rate was 15 mL per hour and each tube contained 5 mL. Pooled fractions with proteinase activity from hydroxylapatite chromatography were concentrated by Amicon® Ultra-15 Centrifugal Filter Devises (Millipore, Inc., Bedford, MA) and dialysed against 50 mM sodium phosphate buffer, pH 7.0. The column (1 by 50 cm) was packed with Bio-Gel P-60 (Bio-Rad, Hercules, CA) and equilibrated with 50 mM sodium phosphate buffer, pH 7.0. The fractions were eluted by the same buffer at the rate of 4 mL per hour. One mL volume was collected for each tube.

Characterization of the purified proteinase. A 12 % Pre Cast Polyacrylamide Electrophoresis Gel (Gradipore™) (Gradipore, Frenchs Forest, Australia) was used to test the purity and homogeneity of the *T. atroviride* proteinase. The gel was run in Tris-glycine-SDS buffer at 150 volts for 90 minutes as described by the manufacturer and stained with silver. The standard protein was low range standard (14.4 kDa to 97.4 kDa) (Bio-Rad, Hercules, CA). A model 111 mini IEF cell with a range of 3-10 (Bio-Rad,

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Hercules, CA) was used to determine the isoelectric point of the proteinase. The proteinase was found in the basic range so an 8-10 pH range was used in the IEF gel. The gel was duplicated and run as described by the manufacturer. One-half was stained with coomassie brilliant blue and one-half was stained with silver.

The effect of plant pathogen mycelia on proteinase expression. Autoclaved mycelia of 0.05 % (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) was added to minimal media consisting of 0.2 % asparagine, 0.2 % $NaNO_3$, 0.05 % $MgSO_4$ $7H_20$, 0.05 % KCl, and 0.1% KH_2PO_4 (38) as the sole carbon source. Each flask contained 50 ml medium and the spore concentration was 10^5 spores/ml. Proteinase was measured after 0, 1, 2, 3, 4, 5, 6, 7, and 8 days incubation. There were three replications for each day measurement and the entire experiment was repeated.

Sensitivity to proteinase inhibitors. Four proteinase inhibitors were dissolved in dimethyl sulfoxide (DMSO) to determine the nature of the purified proteinase. Phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO) is a serine proteinase inhibitor; N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutyamide (E64) (Sigma, St. Louis, MO) is a cysteine proteinase inhibitor; Pepstatin A (Sigma, St. Louis, MO) is an aspartic proteinase inhibitor and 1, 10-phenanthroline (Sigma, St. Louis, MO) is a metallo-proteinase inhibitor. The working solution and procedures used were described by Beynon et al (1).

The effect of pH on proteinase activity. Proteinase activity of the purified preparation was tested at room temperature at (pH 3.6, 4.0, 4.6, 5.0, 5.6, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5,

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9.0, 9.5, 10, 10.5, 11, 12 and 13) in three replications and the experiment was repeated.

Inactivation temperature. The proteinase was treated at (25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C) temperature for 30 min and then tested for proteinase activity at room temperature with three replications. The experiment was repeated.

Antifungal activity of the purified proteinase. The conidia of *Botrytis cinerea* were harvested from a 10-day potato dextrose agar (PDA) culture to test proteinase antifungal activity. Equal aliquots of proteinase solution containing 0.5% glucose and conidia (10^6/mL) were transferred to a microscope slide in a Petri dish lined with wet filter paper, incubated at room temperature (22 °C) for 24 hours and 100 conidia were randomly counted under a microscope to calculate germination rate. Sterile distilled water and 0.5% glucose were used as separate controls. Each treatment had six replications.

N-terminal amino acid sequence. The purified protein was electrobloted to PVDF membrane from SDS-PAGE by using mini trans-blot cell (Bio-Rad, Hercules, CA), and the N-terminal amino acid sequence of the purified proteinase was determined by Midwest Analytical, Inc. (St. Louis, MO).

Results

Proteinase purification. DEAE and CM chromatography purification did not affect the specific activity of the proteinase from *T. atroviride* (Table 4.1) because they cannot bind the proteinase at pH 7.0 even though they could bind contaminating proteins. Specific activity of the proteinase increased during hydroxylapatite chromatography (HAC). The proteinase of interest was eluted when 25 mM sodium phosphate was added 25 mM

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HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)) pH 7.0 buffer. The proteinase was purified by gel filtration approximately 25-fold compared to the starting level.

Characterization of the purified proteinase. The molecular weight of the purified proteinase is approximately 18.8 kDa was estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4.1) and the pI is approximately 8.5 as determined by isoelectric focusing. The proteinase *T. atroviride* had optimal activity at two pH ranges (Figure 4.2) with the greatest activity at pH 9.0. Activity was greatly reduced after 30 minutes treatment at 50 °C. Therefore, the inactivation temperature was determined to be 50 °C (Figure 4.3). Dimethyl sulfoxide and 50 mM pH 7.0 phosphate buffer served as negative controls. Proteinase activity was significantly inhibited by 1 mM PMSF (a serine proteinase inhibitor). E64 (0.1 mM) (a cysteine proteinase inhibitor), 1 μg/ml pepstatin A (an aspartic proteinase inhibitor) and 1mM 1,10-phenanthroline (a metallo-proteinase inhibitor) had no effect on proteinase activity (Figure 4.4). The purified proteinase was identified as a serine proteinase.

Effect of mycelia of fungal pathogens on proteinase production by *T. atroviride*. Proteinase activity was detected with fungal mycelia and milk but not with glucose (Figure 4.1). The induction time required for proteinase production depended on the pathogen mycelia. Specific proteinase activity peaked 1-3 days after *T. atroviride* was grown in the minimal media supplemented with 0.05 % autoclaved mycelia of the pathogens (Figure 4.5) with proteinase induced by mycelia of *R. solani* and *S. sclerotiorum* highest the first day. Specific proteinase activity was highest the second day

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with *P. capsici* and the third day with *B. cinerea.*

Antifungal activity. *T. atroviride* proteinase significantly inhibited conidial germination of *B. cinerea* and germinating tubes were also shorter than those in glucose 95.7 % germination and water 14 % germination (Figure 4.6). Only 12.7 % of the conidia germinated in 0.5 % glucose plus 0.5 mU purified 18.8 kDa proteinase placing the inhibition rate around 80-86%.

N-terminal amino acid sequence. The N-terminal amino acid sequence of the 18.8 kDa proteinase determined by MIDWEST ANALYTICAL, INC (St. Louis, MO) was IVGGTTAAAG. A database homology search using the BLASTp algorithm showed the greatest similarity to an extracellular protease from *Cochliobolus carbonum* (29).

Discussion

Trichoderma strains produce several proteinases when grown in liquid media (9, 11, 41). Two proteinase peaks were observed during gel filtration. The second peak was identified as the 18.8 kDa proteinase. In the first peak, at least two protein bands were found with SDS-PAGE molecular weights between 30 to 45 kDa. This shows that *T. atroviride* produces at least two different size proteinases. Further characterization and function of the other proteinases are needed.

Proteins are components of the cell wall of pathogenic fungi and proteinases produced by *Trichoderma* may be involved in the parasitism of plant pathogenic fungi. Pretreating mycelia of *Fusarium oxysporum* with proteinase makes the mycelia more susceptible to chitinase and β-glucosidase (33). The culture filtrate of *T. harzianum* T39, a poor

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chitinase and β-glucanase producer *in vitro*, had high proteinase activity, inhibited germination and shortened germ tube length of *B. cinerea* on the surface of bean leaves, partially deactivated hydrolytic enzymes (endo- and exo-polygalacturonase) produced by *B. cinerea* and reduced disease severity (13). These results suggest that proteinases are involved in antifungal activity. Purified proteinases also may be involved in disease control by *Trichoderma*. A 31 kDa basic serine proteinase from *T. harzianum* has been purified and high levels of proteinase Prb1 activity have been detected during mycoparasitism (13, 15, 16, 18). Transgenic *Trichoderma* lines with multiple copies of the *prb1* gene provided much better control *R. solani* in cotton. These results indicate that Prb1 plays an important role during parasitism by *T. harzianum* (16). It was suggested that the 31 kDa proteinase (Prb1) degraded phytopathogen cell walls and membranes with the released proteins after lysis available as nutrients to mycoparasites (16). A purified 18.8 kDa proteinase from *T. harzianum* 1051 hydrolyzed the cell wall of the phytopathogenic fungus *Crinipellis perniciosa* after 48 hours contact (11) to indicate involvement of hydrolytic enzymes in the antagonistic process. A serine proteinase from *T. virens* significantly stimulated terpenoid production in cotton and increased peroxidase activity in cotton radicles (19). The 18.8 kDa serine proteinase reported in this paper also may be involved in the biocontrol of phytopathogenic fungi by *T. atroviride* since autoclaved mycelia of plant pathogens can be used as the only carbon source and the proteinase has strong antifungal activity by inhibiting germination of *B. cinerea* conidia. Cell wall components of fungi vary by species and growth stages (5) which may explain why the 18.8 kDa proteinase was produced after different incubation times with

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autoclaved pathogen mycelia. I did not determine which mycelia components induced proteinase in this study.

The first 10 N-terminal amino acids of the reported 18.8 kDa proteinase are IVGGTTAAAG. It is similar to a 27 kDa trypsin proteinase from *T. harzianum* according to a database homology search (29). The first 20 N-terminal sequence residues of another serine trypsin-like proteinase from *T. viride*, pI 7.3, with a molecular weight of 25 kDa are IVGGTAAAAGEFPPIVSLQN (36). Only one of first 10 N-terminal sequence residues is different from the *T. atroviride* proteinase reported here. The differences in these two proteinases include differences in molecular size, and the 25 kDa proteinase was only weakly inhibited by PMSF and did not show any antifungal activity (36). The N-terminal amino acid sequence of the other reported 18.8 kDa proteinase is VPSTVWTVCP (11); therefore, it is not similar in homology at the N-terminal and to the proteinase from *T. atroviride* reported here. The N-terminal amino acid sequence of *T. atroviride* is also different from the DTVSYDTGYDNGSRSLNDV sequence of the serine proteinase of *T. virens* (19).

Extracellular enzymes (such as chitinases, β-glucanases and proteinase) produced by *Trichoderma* and *Gliocladium* depend on the carbon source (laminarin, chitin) (33, 35), age of the culture (35), cell wall of pathogens (33, 35) and pH (9). Nitrogen sources such as yeast extract, peptone and casein induce acidic proteinases. Alkaline and neutral proteinases are induced by less readily digested carbon sources (lactose or chitin), carbon starvation, and some organic nitrogen sources (casein) (9). The 31 kDa proteinase from *T. harzianum* was induced by autoclaved mycelia, a fungal cell wall preparation or chitin,

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but was not induced by glucose (15). The 18.8 kDa serine proteinase reported in this paper also was induced by autoclaved mycelia of *B. cinerea, P capsici, R. solani* and *S. sclerotiorum* and milk. There were no proteinase bands when glucose was added to the minimal media (Figure 4.1). The timing of proteinase production varied with the inducing pathogen mycelia and may be related to different molecular architectures of hyphal cell walls (5).

Glucose regulated extracellular enzyme production by *Trichoderma* (6, 14, 37) in that it repressed chitinase (14, 37), β-1,3-glucanase (6, 14), alkaline proteinase PRB1 (15) and aspartyl proteinase production (10); however, glucose is reported to induce higher acid proteinase than the cell wall of *R. solani* and chitin (35). The 18.8 kDa proteinase produced by *T. atroviride* was inhibited by 0.05 % glucose and no proteinase bands appeared when glucose was added to the minimal media (Figure 4.1).

In summary, the18.8 kDa proteinase produced by *T. atroviride* is a basic serine proteinase, induced by autoclaved mycelia of several fungal plant pathogens, inhibited by glucose (0.05 %) and strongly inhibits conidial germination of *B. cinerea*. Further studies are needed to assess the role of the 18.8 kDa serine proteinase in the actual control of plant pathogenic fungi. In future research, degenerative primers corresponding to the N-terminal amino acid sequence of this proteinase may be used to develop probes to specifically determine expression levels of the gene encoding this protein under biocontrol conditions. Finally, the gene encoding the 18.8 kDa serine proteinase may be isolated using the N-terminal amino acid sequence information. Isolation of the gene would facilitate studying its overexpression or underexpression in transgenic

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Trichoderma. Further characterization in this manner may help determine the role of the 18.8 kDa serine proteinase in the control of plant pathogenic fungi by *T. atroviride*.

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Table 4.1. Purification of proteinase from *T. atroviride.*

Steps	Volume (mL)	Total protein (mg)	Total activity (U)	Sp act[a] (U/mg)	Yield (%)	Purification (fold)
PEG[b]	75	49.9	2.76	0.055	100	1
DEAE[c]	71	43.6	2.02	0.046	73.2	0.84
CM[d]	64	38.9	1.87	0.048	67.8	0.87
HAT[e]	35	0.93	0.26	0.28	9.42	5.09
GF[f]	3.6	0.05	0.07	1.401	2.53	25.47

Note: a: Specific Activity; b: polyethylene glycol; c: Diethylaminoethyl (DEAE) chromatography; d: carboxymethyl (CM) cellulose chromatography; e: hydroxylapatite chromatography; f: gel filtration.

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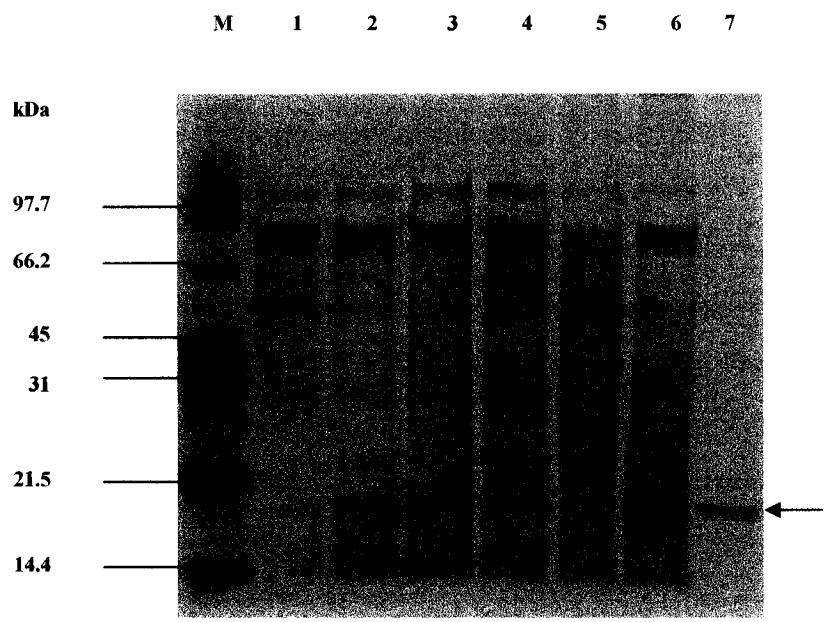

Figure 4.1. SDS-PAGE stained with silver. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) concentrated of proteins in culture filtrates of *T. atroviride* stained with silver showing the effect of pathogen mycelia production of the 18.8 kDa proteinase. Lane M: 2.4 μg of low range molecular weight size marker. Lane 1: 1.3 μg of proteins in minimal media supplemented with 0.05 % autoclaved mycelia of *P. capsici*. Lane 2: proteins in minimal media supplemented with 0.05 % autoclaved mycelia of *S. sclerotiorum*. Lane 3: proteins minimal media supplemented with 0.05 % autoclaved mycelia of *R. solani*. Lane 4: proteins in minimal media supplemented with 0.05 % autoclaved mycelia of *B. cinerea*. Lane 5: proteins in minimal media supplemented with 0.05 % glucose. Lane 6: proteins in minimal media supplemented with 0.05 % skim milk. Lane 7: 1.0 μg of purified proteinase.

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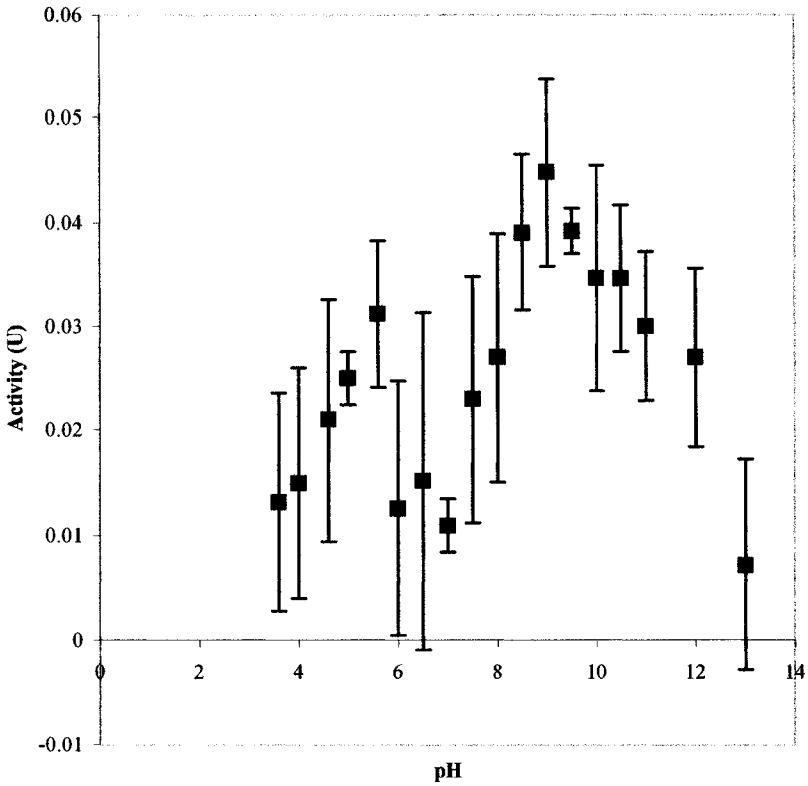

Figure 4.2. The effect of pH on proteinase activity of *T. atroviride*. Fifty microliter samples of purified proteinase were suspended in 50 mM potassium acetate buffer (pH 3.6-5.6), 50 mM potassium phosphate buffer (pH 6.0-8.0), or 50 mM Tris base buffer (pH 8.5-13). Proteinase assays used azocasein as the substrate. Assays at each pH were performed in three replications and the entire experiment was repeated. The bars show the combined results of two experiments. Error bars indicate standard deviations.

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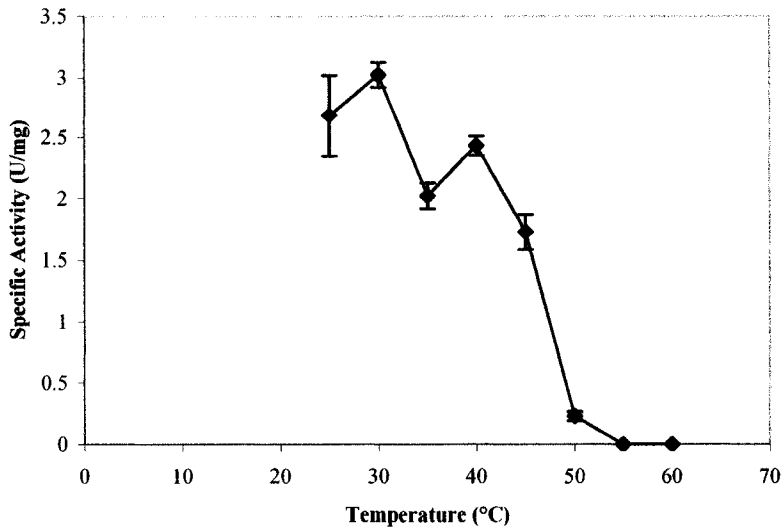

Figure 4.3. Inactivation temperature of the purified *T. atroviride* proteinase. Fifty microliter samples of purified proteinase were treated at the respective temperature for 30 minutes before assaying for proteinase activity using azocasein as the substrate. Assays at each temperature were performed in three replications and averaged. Results were the same for the repeated experiment (data not shown). Error bars indicate standard deviations.

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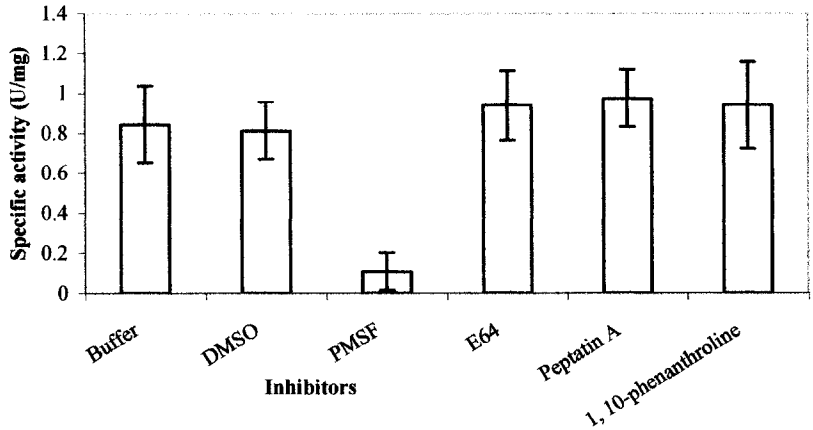

Figure 4.4. The effect of proteinase inhibitors on proteinase from *T. atroviride*. The proteinase assay mixture includes 445 µl of 50 mM potassium phosphate buffer, pH 7.0, 50 µl of purified enzyme and 5 µl of inhibitor, DMSO or buffer. The mixture was incubated at 30 °C for one hour, then 100 µl of 2 % azocasein was added and the materials were incubated at 25 °C for 30 minutes before 1.2 mL of 5 % trichloroacetic acid (TCA) was added to the mixture which was centrifuged at 12,000 rpm for 5 minutes. The supernatant (1.2 mL) and 0.8 mL of 1 M sodium hydroxide were mixed in a cuvette and measured at 440 nm. The experiment had three replications. DMSO = dimethyl sulfoxide. Buffer and DMSO served as negative controls. The final concentration of PMSF and the final concentration of 1, 10- phenanthroline in the mixture are 1 mM. The final concentration of E64 in the mixture is 0.1 mM and the final concentration of Pepstatin A is 1 µg/ml. Error bars indicate the standard deviation.

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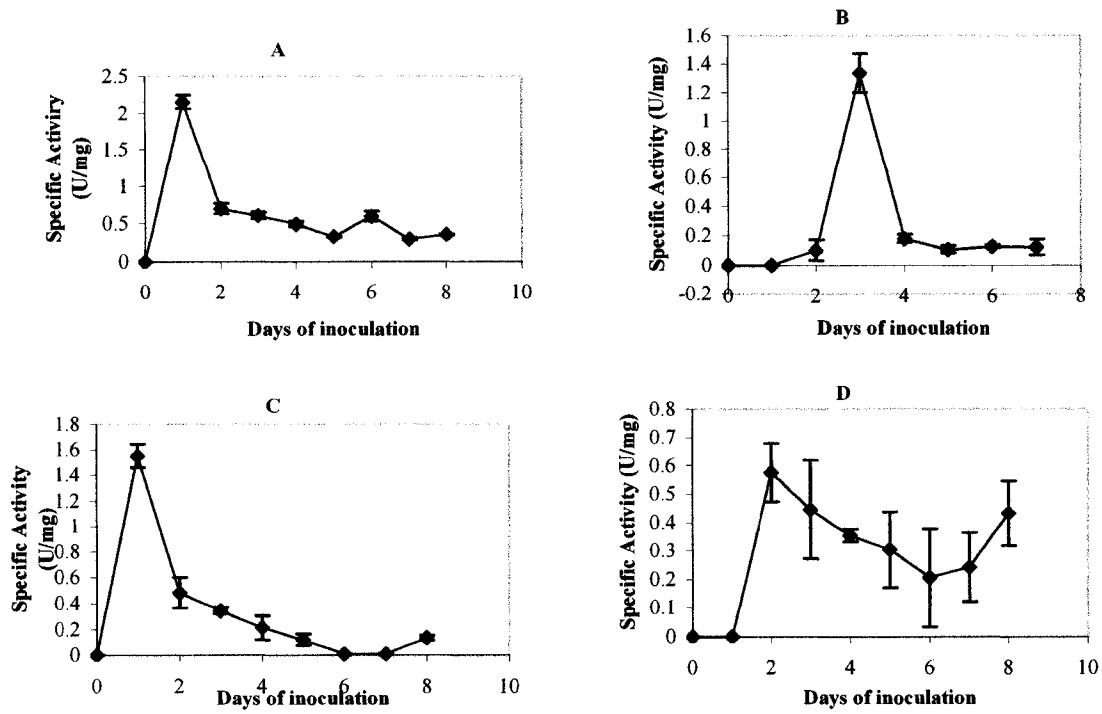

Figure 4.5. Timing of *T. atroviride* proteinase production. Proteinase induced by mycelia from different pathogens. A, minimal media supplemented with 0.05 % autoclaved mycelia of *S. sclerotiorum* as a sole carbon source. B, minimal media supplemented with 0.05 % autoclaved mycelia of *B. cinerea* as sole carbon source. C, minimal media supplemented with 0.05 % autoclaved mycelia of *R. solani* as the sole carbon source. D, minimal media supplemented with 0.05 % autoclaved mycelia of *P. capsici* as the sole carbon source. Each treatment had three replications and the entire experiment was repeated. The specific activity was different between two experiments but the pattern was the same (data not show). Error bars indicate the standard deviation.

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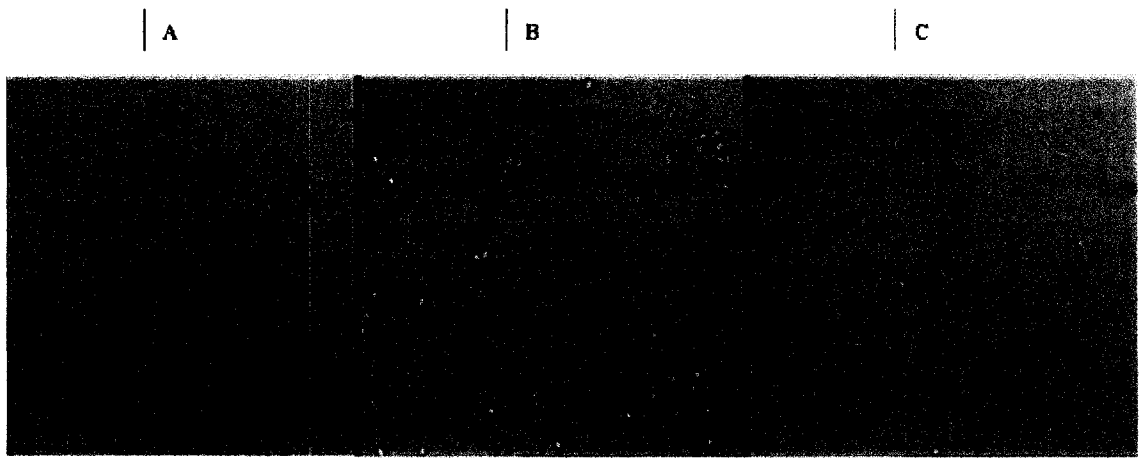

Figure 4.6. Conidial germination of *B. cinerea.* Conidia of *Botrytis cinerea* were harvested from a 10-day PDA culture. Equal aliquots of proteinase solution containing 0.5% glucose and conidia solution (10^6/mL) were transferred to microscope slides, placed in a Petri dish, kept humid with wet filter paper, incubated at room temperature (22 °C) for 24 hours and then randomly counted under the microscope 100 conidia to calculate the germination rate. Each treatment had six replications. A, in 0.5 % glucose. B, in 0.5 % glucose plus 0.5 mU purified 18.8 kDa proteinase and C in sterile distilled water.

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CHAPTER 5: CHARACTERIZATION OF AN ENDO-β-1,3-GLUCANASE ASSOCIATED WITH BIOCONTROL ACTIVITY OF A COLD TOLERANT *TRICHODERMA ATROVIRIDE*

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Abstract

A 77 kDa endo-β-1,3-glucanase was purified from cold tolerant *T. atroviride* 901. The purified endo-β-1,3-glucanase showed strong antifungal activity by inhibiting spore germination of *Botrytis cinerea*. The endo-β-1,3-glucanase had activity against laminarin even at low temperature (6 °C and 15 °C). The optimal temperature for the β-1,3-glucanase was 45 °C, and optimal pH was 4.0. The enzyme was active against β-1,3 linkage polymers and is a β-1,3-glucanase although it differs in only one N-terminal amino acid from the sequence reported for α-1,3-glucanase. The Km of this endo-β-1,3-glucanase was 6.2 mg/ml which is higher than other reported β-1,3-glucanases.

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Introduction

There are two types of β-1,3-glucanases, one is exo-β-1,3-glucanase [EC.3.2.1.58] and the other is endo-β-1,3-glucanase [EC.3.2.1.39] (7). β-1,3-glucanases are involved in biocontrol bacteria by (3,16) and fungi (4,6,7,12,15,17,19,30,31,36,37,38,39). β-1,3-glucanases also found in plants as a PR protein to induce host resistance to pathogens (2,8,24). Elucidation of the production and function of β-1,3-glucanase of biocontrol agents can provide a better understanding of the mechanisms of biocontrol by *Trichoderma.*

Trichoderma has been widely studied for the biocontrol of plant pathogenic fungi (7). *Trichoderma* controls plant diseases by competition, antibiosis, inducing host resistance and producing cell-degrading enzymes (21). Some cell-wall degrading enzymes such as chitinases, glucanases, and proteinases show direct antifungal activity (1,7,12,14,15,22,23,36,37). β-1,3-glucanase was an important cell-wall degrading enzyme involved in mycoparasitism by *Trichoderma* because β-glucan is a major part of the cell wall of fungi, especially groups belonging to the Oomycetes (11). Several β-1,3-glucanases have been purified and characterized from *Trichoderma* recently (4,12,36,37). However, there are fewer studies of β-1,3-glucanase than the chitinases produced by *Trichoderma.*

Trichoderma atroviride is a cold tolerant species and can parasitize a wide range of plant pathogenic fungi (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) (41). It produces chitinases, β-1,3-glucanase, and proteinases

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(10). Purification and characterization of a β-1,3-glucanase from *T. atroviride* 901C reported in this chapter is important to understand the mechanisms of biocontrol by *T. atroviride*.

Materials and Methods

The over-all production and purification steps for β-1, 3-Glucanase from *T. atroviride* are presented in Table 5.1 and Figure 5.1.

Chemicals and Reagents. Carboxymethyl cellulose, corn starch, dextran, soluble laminarin (from *Laminaria saccharina*), pullulan, xylan (from oat spelts), and ρ-Nitrophenyl β-D-glucopyranoside were obtained from Sigma Chemical Co., (St. Louis, MO). Pustulan (from *Umbilicaria papullosa*) was obtained from Calbiochem-Behring (La Jolla, CA).

Induction of β-1,3-glucanase production. *Trichoderma atroviride* biotype 901, an excellent biocontrol agent, was used throughout this study. β-1,3-glucanase was produced by incubating 10^5 spores/ml of *T. atroviride* into 200 ml potato dextrose broth media containing 6 % glucose and incubating at room temperature for one day on a shaker at 135 rpm.. The mycelia were collected on a 0.45 μm filter (Millipore, Bedford, MA), placed into 1000 ml of 0.5 % autoclaved mycelia of *Phytophthora capsici* and incubated for 5 days on a reciprocal shaker (135 rpm) at room temperature.

β-1,3-glucanase activity assay. Laminarin (Sigma, St. Louis, MO) was used as the substrate to assay for activity of endo-β-1,3-glucanase (13,25,34,42). Briefly, 400 μl of reaction mixture containing 80 μl of sample solution, 160 μl of 50 mM pH 5.0 acetate

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buffer and 160 µl of 0.5 % laminarin in 50 mM pH 5.0 acetate buffer was incubated at 37 °C for 30 minutes before adding 1 milliliter of solution A (Solution A contained 40.0 g/L Na_2CO_3, 16.0 g/L glycine and 0.45 g/L $CuSO_4$) and 1 milliliter of solution B (Solution B contained 0.12 g/L neocuproine hydrochloride in 100 mL) into the reaction mixture (13,25,34). This was autoclaved for 10 minutes to develop the diagnostic color (42). Samples boiled for 5 minutes served as a control. Absorbance was measured at 440 nm. One unit of β-1,3-glucanase was defined as 1 milliliter of enzyme producing 1 micromole glucose per minute (12,13,25,34).

Protein determination. Protein concentration was determined with 30 % Bradford reagent (Sigma, St. Louis, MO). Fifty microliters of enzyme preparation was added to 2 ml of 30 % Bradford reagent and was incubated for 30 minutes at room temperature before measuring absorbance at 595 nm. Bovine serum albumin (BSA) was used as a protein standard.

Purification of β-1,3-glucanase

Filtrate concentration. The fungal filtrate was transferred into 600-800 kDa cut-off dialysis tubing with polyethylene glycol (PEG 8000) (VWR, Bristol, CT) outside to concentrate it 20 times (22,23).

DEAE chromatography. The concentrated samples were then dialysized in 50 mM, pH 7.0, potassium phosphate buffer overnight and applied to a 20 cm DEAE cellulose (Sigma, St. Louis, MO) column (pre-equilibrated with 50 mM, pH 7.0, potassium phosphate buffer). The sample was eluted from the column with 25 ml of 50 mM, pH 7.0, potassium phosphate buffer, 25 ml of 50 mM potassium phosphate buffer containing 100

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mM sodium chloride, 25 ml of 50 mM potassium phosphate buffer containing 200 mM sodium chloride and 50 mM potassium phosphate buffer containing 1 M sodium chloride, respectively. The fractions were collected at 5 ml per tube at the rate of 1 ml/minute.

Phenyl sepharose chromatography. Fractions containing β-1,3-glucanase activity were pooled and dialysed in distilled water for one day and then in 50 mM, pH 5.0, potassium acetate buffer containing 1.5 M ammonium sulfate overnight. The sample was applied to a 15 mL phenyl sepharose™ high performance (Amersham Biosciences, Uppsala, Sweden) column (pre-equilibrated with 50 mM, pH 5.0, potassium acetate buffer containing 1.5 M ammonium sulfate). The column was eluted with 50 ml of 50 mM, pH 5.0, potassium acetate buffer containing 1.5 M ammonium sulfate. The column was then eluted with down degradation from 50 ml of 50 mM, pH 5.0, potassium acetate buffer containing 1.5 M ammonium sulfate to 50 ml of 5 mM, pH 5.0, potassium acetate buffer (9). The fractions were collected at 5 ml per tube at the rate of 0.5 ml per minute.

Chromatofocusing chromatography. The fractions with β-1,3-glucanase activity from the previous procedure were pooled and dialysed in distilled water for one day and in 25 mM, pH 9.4, ethanolamine-hydrochloride (Sigma, St. Louis, MO) overnight. The sample was applied to a 40 cm column pre-equilibrated with 25 mM, pH 9.4, ethanolamine-hydrochloride polybuffer exchanger 94 (PBE 94) (Sigma, St. Louis, MO). The sample was eluted with 10 times dilution, pH 6.0, polybuffer 96-hydrochloride (Sigma, St. Louis, MO) at 1 mL per minute with 5 ml fractions were collected. The fractions with β-1,3-glucanase activity were pooled for further use.

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Characterization of β-1,3-glucanase

Molecular weight determination. The molecular weight of β-1,3-glucanase was determined using 12 % pre-cast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Gradipore™, Frenchs Forest NSW, Australia).

Km and Vmax. The concentrations of laminarin used to test β-1,3-glucanase activity were 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1mg/ml, 0.5 mg/ml and 0 mg/ml. Km and Vmax were determined by A Lineweaver-Burk plot (26).

pH effect. Citric acid and sodium phosphate were used to make 100 mM, pH 2.2 to pH 8.0 buffers. 100 mM, pH 9.0 to pH 12.38 buffers were made with sodium borate. Each pH treatment had three replications and was repeated once. β-1,3-glucanase activity was determined according to the procedure described above.

Temperature effect. β-1,3-glucanase activity was determined at 7 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C and 65 °C with a assay as described above. Each temperature treatment had three replications and was repeated once.

Inactivation temperature. β-1,3-glucanase was incubated for 30 minutes at different temperatures from 15 °C to 65 °C and the activity was then determined by performing the assay as describe above. Each temperature treatment had three replications and was repeated once.

Substrate specificity of purified β-1,3-glucanase. Various glucans and xylans of different linkage types were selected to test the substrate specificity of the purified β-1,3-glucanase (Table 5.2). The enzyme assay was conducted in the standard manner by using 0.5 % (wt/vol) substrate.

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Antifungal activity. Twenty microliters of *Botrytis cinerea* spores (1.5×10^7/ml) were put into 0.5 % glucose, 0.5 % glucose plus 20 µl of the purified β-1,3-glucanase, 0.5 % glucose plus 20 µl of the heated purified β-1,3-glucanase and distilled water, respectively. The spores were incubated at room temperature for 24 hours and the germination was then calculated by randomly counting 100 spores under a microscope. Each treatment had five replications.

N-terminal amino acid determination. The purified protein was electrobloted to PVDF membrane from SDS-PAGE by using mini trans-blot cell (Bio-Rad, Hercules, CA), and the N-terminal amino acid sequence determination was conducted by Midwest Analytical (St. Louis, MO).

Results

Purification of β-1,3-glucanase. Table 5.1 showed the purification steps of β-1,3-glucanase from *T. atroviride*. β-1,3-glucanase was purified about 12 fold with a final yield of 23 %. DEAE does not bind β-1,3-glucanase, so that the β-1,3-glucanase was eluted at the beginning of chromatography and the specific activity did not change. Phenyl sepharose bound the β-1,3-glucanase and it was eluted from the column when 5 mM potassium phosphate buffer was applied. β-1,3-glucanase was purified three times in this manner, during chromatofocusing the purified β-1,3-glucanase was eluted from pH 7.4 to 7.3 with a final purification of 12 fold.

Properties of the purified β-1,3-glucanase. Molecular weight and pI value β-1,3-glucanase. The molecular weight of the β-1,3-glucanase from *T. atroviride* was about

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77.8 kDa based on SDS-PAGE (Figure 5.1). The pI value was 7.3-7.4 as determined by chromatofocusing.

pH optimum. The β-1,3-glucanase from *T. atroviride* was active from pH 2 to 7 and had the highest activity at pH 4 (Figure 5.2). β-1,3-glucanase lost its activity above pH 8.0 (Figure 5.2). The activities of β-1,3-glucanase at pH 6 and pH 7 were significantly lower than at pH 3-5.

Temperature optimum and inactivation temperature. β-1,3-glucanase activity was low at 6 °C, peaked at 45 °C, with no significant difference between 15 °C and 55 °C, decreased from 60 °C with little activity at 65 °C (Figure 5.4), and was inactive after treatment at 50 °C for thirty minutes (Figure 5.3).

Km and Vmax. The Km of purified β-1,3-glucanase was 6.25 mg/ml by a Lineweaver-Burk plot. Vmax was 3.12 mg/ml/min.

Substrate specificity. β-1,3-glucanase from *T. atroviride* was active on substrate only containing β-1,3 linkages (Table 5.2) with the highest activity against laminarin and glucan. There was no activity against the other substrates tested.

N-terminal determination. The first eleven N-terminal amino acid sequence of the purified β-1,3-glucanase was ASSADRLVFXH. The sequence was similar to a mutanase (GenBank/EBI accession no. AF214480 and AAF27911) and a α-1,3-glucanase(1).

Antifungal activity. The purified β-1,3-glucanase significantly inhibited spore germination of *B. cinerea*. Almost all of the spores germinated in 0.5 % glucose solution, but fewer spores geminated in purified β-1,3-glucanase plus 0.5 % glucose. The conidia of *B. cinerea* also germinated in water and in heated β-1,3-glucanase plus 0.5 % glucose,

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but the germination rate was lower under these conditions than in the 0.5 % glucose solution (Figure 5.5).

Discussion

The seven β-1,3-glucanases produced by *Trichoderma* under inducing conditions are complicated (39). At least two β-1,3-glucanase bands were detected from *T. atroviride* 901 by SDS-PAGE (date not shown), and three β-1,3-glucanase bands appeared on the IEF gel (data not shown).

There are two types of β-1,3-glucanases. One is endo-β-1,3-glucanase and the other is exo-β-1,3-glucanase (7). The purified 77 kDa enzyme was classified as an endo-β-1,3-glucanase by its inability to hydrolyze ρ-Nitrophenyl-β-D-glucopyranoside (4,27).

Chitin and glucan are two major components of plant pathogenic fungi (11). Chitinases and glucanases play a significant role in the biocontrol of fungal pathogens by *Trichoderma*, and these enzymes have direct antifungal activity (12,22,35,36,37). The purified β-1,3-glucanase from *T. atroviride* also showed antifungal activity by inhibiting spore germination of *B. cinerea.*

One function of β-1,3-glucanase was to reduce osmotic stress and prevent vesicle rupture at low temperatures (20). *T. atroviride* 901 is a cold tolerant strain and produces β-1,3-glucanase at low temperatures (7 °C) (data not shown). The activity of β-1,3-glucanase at 6 °C was 0.29 U and about 22 % of its activity at 45 °C. The activity of β-1,3-glucanase at 15 °C was about 77 % of the highest activity at 45 °C. These results confirm that cold-

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tolerant *T. atroviride* 901 has antifungal activity and can control pathogenic fungi at low temperature (3 °C-10 °C) (41).

The first ten N-terminal of amino acid sequence of purified β-1,3-glucanase was similar to a α-1,3-glucanase associated with *T. harzianum* biocontrol and only one amino acid (the tenth) was different (1). Substrates specificity showed that the β-1,3-glucanase reported here was active against laminarin and glucan while the α-1,3-glucanase from *T. harzianum* was negative against laminarin and glucan (1). The β-1,3-glucanase purified from *T. atroviride* was not α-1,3-glucanase.

The molecular weight of the purified enzyme was 77.8 kDa according to SDS-PAGE, and similar to the 78 kDa β-1,3-glucanase previously reported (12); however, the pI value of this β-1,3-glucanase was about 7.3 to 7.4 according to chromatofocusing. It was between pI 8.0 of a 78 kDa endo-β-1,3-glucanase and pI 6.2 of a 78 kDa exo-β-1,3-glucanase (7). The optimal pH was 4.0, the optimal temperature was 45 °C and the inactivation temperature was 50 °C. The Km of this enzyme was higher than those from other *Trichoderma*. Thus, the purified β-1,3-glucanase was different from other *Trichoderma* β-1,3-glucanases (12,36). Its activity against laminarin at 6 °C confirms that *T. atroviride* 901 is a cold active strain and can parasitize plant pathogens at low temperature. Having similar N-terminal amino acids with a reported α-1,3-glucanase, but positive against laminarin and glucan show the 1,3-glucacanase reported here is a β-1,3-glucanase. Further studies will focus on determining the whole DNA sequence of β-1,3-glucanase and compare difference obtained with the whole sequence of α-1,3-glucanase.

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TABLE 5.1. Purification of β-1,3-glucanase from *T. atroviride*

Purification step	Vol (ml)	Protein (mg)	Activity (U)	Sp act[a] (U/mg)	Purification (fold)	Yield (%)
Filtrate concentration	55	4	51.22	12.79	1	100
DEAE[b]	60	2.15	27.90	12.95	1.01	54.47
Phenyl-Sepharose	26	0.66	26.92	40.93	3.20	52.56
Chromatofocusing	21.8	0.074	11.89	160.43	12.54	23.21

a: Specific activity

b: Diethylaminoethyl cellulose

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TABLE 5.2. Substrate specificity of purified β-1,3-glucanase from *T. atroviride*

Substrate[a]	Monomer	Linkages	Relative β-1,3-glucanase activity (%)[b]
Laminarin (*L. digitata*)	Glucose	β-1,3, β- 1,6	100
Pullulen (*A. pullulans*)	Glucose	α-1,4, α-1,6	0
Glucan (*S. cerevisiae*)	Glucose	β-1,3, β-1,6	62.7
Lichenan(*C. islandica*)	Glucose	β-1,3, β-1,4	0
Dextran	Glucose	α-1,6	0
Pustulan(*U. papullosa*)	Glucose	β-1,6	0
Carboxymethyl cellulose	Glucose	β-1,4	0
Glycol chitosan	N-glucosamine	β-1,4	0
Chitosan	N-glucosamine	β-1,4	0
Soluble starch	Glucose	α-1,4, α-1,6	0
Xylan (*P. dumetosus*)	Xylose	β-1,4	0
ρ-Nitrophenyl β-D-glucopyranoside	glucopyranoside	Exo-β-1,3	0

[a]All substrates (except ρ-Nitrophenyl β-D-glucopyranoside) were used at a concentration of 0.5 %.

[b]Each value is the mean of three replications.

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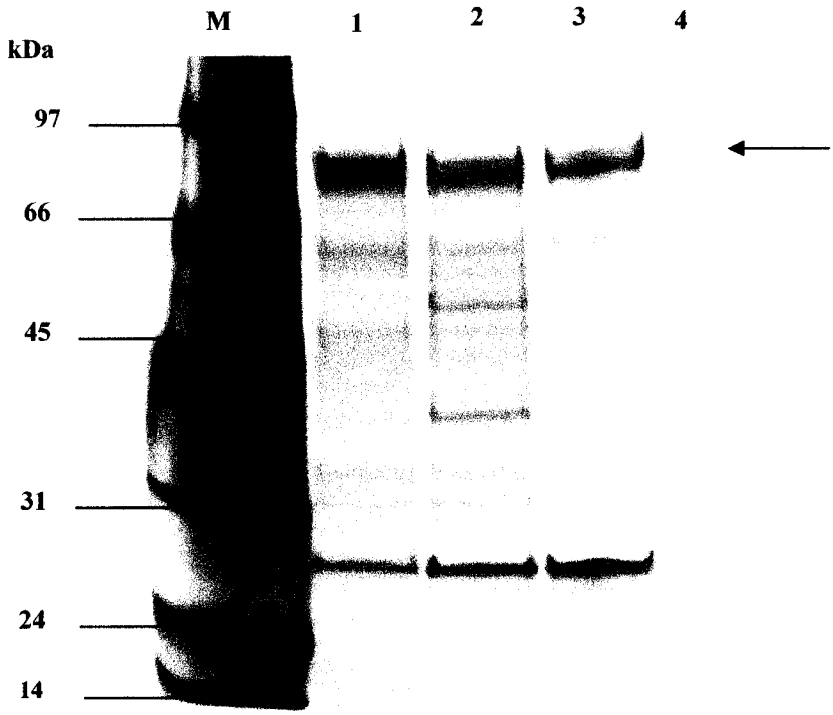

Figure 5.1. SDS-PAGE stained with silver. The gel showed proteins from different purification steps of β-1,3-glucanase from *T. atroviride*. Lane M, low molecular weight marker; lane 1, proteins after concentration of filtrate; lane 2, proteins from DEAE chromatography fractions; lane 3, proteins from phenyl Sepharose chromatography fractions; lane 4, proteins from chromatofocusing. (Arrows shows β-1,3-glucanase protein.)

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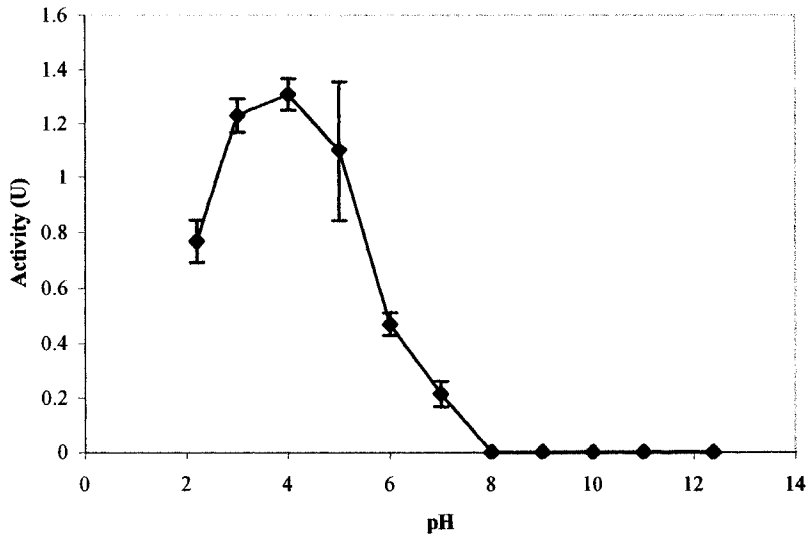

Figure 5.2. The effect of pH on β-1,3-glucanase activity. β-1,3-glucanase activity was measured at different pHs. Each pH treatment had three replications was and repeated once. Although slightly different values were obtained at the different pHs between the two experiments, they showed the same activity pattern. The values shown are the average of three replications. The error bars are the standard deviations.

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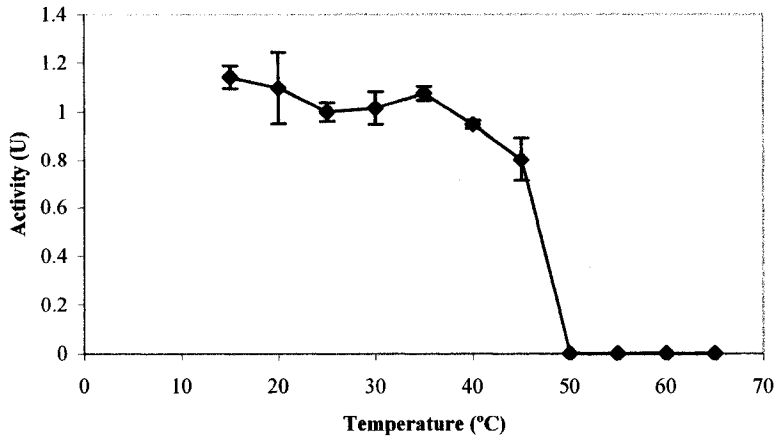

Figure 5.3. The inactivation temperature. The purified β-1,3-glucanase after incubation at each temperature for thirty minutes and the tested the activity. The value is the average of three replications and the one experiment was repeated once with similar results. The bars are the standard errors.

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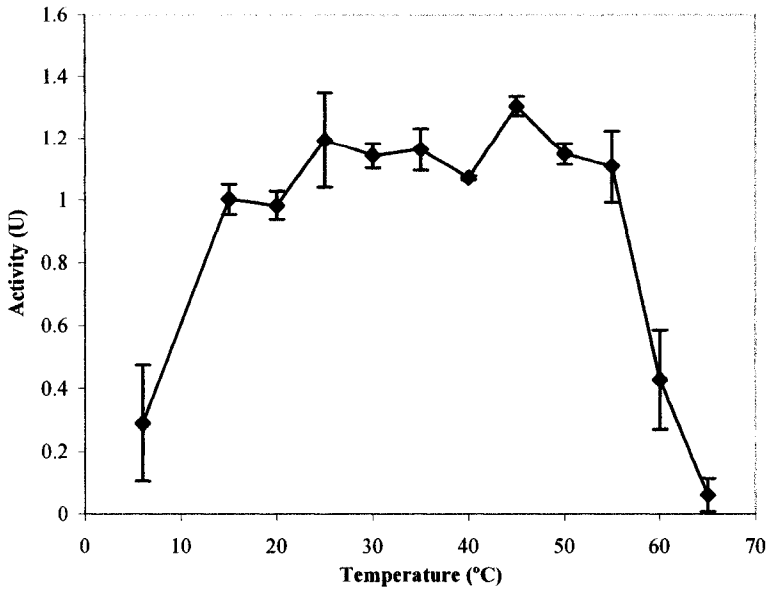

Figure 5.4. The temperature optimum. The purified β-1,3-glucanase was tested the activity at different temperature. The values in the figure are averages of three replications. The experiment was repeated once with the same pattern. The error bars show the standard deviations of each treatment.

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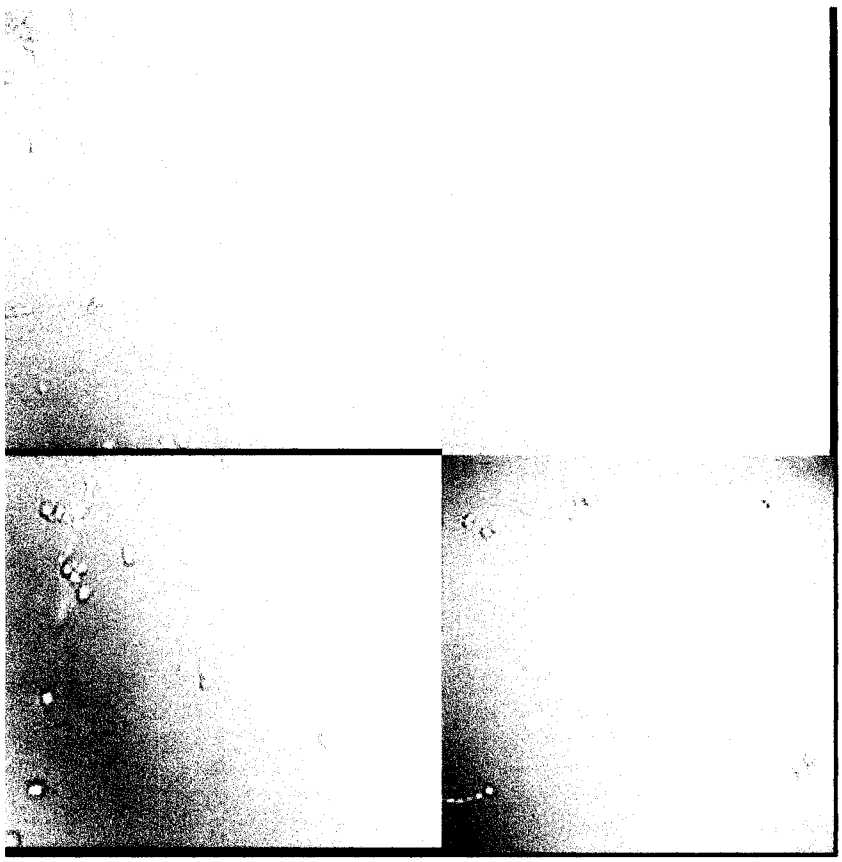

Figure 5.5. The inhibition of *B. cinerea* spore germination by purified β-1,3-glucanase. Upper left, spore germination in 0.5 % glucose; upper right spore germination in 0.5 % glucose plus purified β-1,3-glucanase; lower left spore germination in 0.5 % glucose plus heat denatured purified β-1,3-glucanase; lower right spore germination in sterile water.

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CHAPTER 6: THE 73 kDa β-ACETYL-D-GLUCOSAMINIDASE GENES ASSOCIATED WITH COLD TOLERANT *TRICHODERMA ATROVIRIDE*

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Abstract

Three 73 kDa β -N-acetylglucosaminidase genes were amplified from cold tolerant *T. atroviride* biotype 861, 603, and 453 by Polymerase Chain Reaction (PCR). The amplified product was about 2.7 kb. Sequences of the genes for 861, 603 and 453 were conducted by forward and reverse orientations and 2416, 2418 and 2564 base pairs were obtained from 603, 453 and 861, respectively. Eleven unique base pairs for *T. atroviride* biotypes were identified by comparing them with the published β -N-acetylglucosaminidase gene from GeneBank. Comparison with the published gene showed the number of base pair insertions was 4, 5 and 5, and single base pair change was 13, 13, and 9 for 603, 453 and 861, respectively. The genes of these three cold tolerant *T. atroviride* biotypes are different from the published gene of *T. harzianum.* Similarity of the two mutants 603 and 453 were high and they were distinct from wild type 861. Single and multiple nucleotide polymorphism may be used to determine the phylogenetic similarities of different *Trichoderma spp.* and strains.

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Introduction

Trichoderma species have been used as biocontrol agents to control plant pathogenic fungi (12). The mechanisms involved in biocontrol with *Trichoderma* include competition, antibiosis, mycoparasitisms and induced systemic resistance of plants (7, 8, 10). Cell wall degrading enzymes produced by *Trichoderma* play a significant role in mycoparasitisms (8, 10). Chitin and glucan are major components of the cell wall of plant pathogenic fungi (5) and chitinases and β-glucanases are important cell wall degrading enzymes associated with *Trichoderma* biocontrol (1, 11).

Three groups of chitinases are associated with *Trichoderma* biocontrol, e.g., β-N-acetyl-D-glucosaminidase (EC 3.2.1.52), chitobiosidase (exochitinase), and endochitinase (EC 3.2.1.14) (11). Four enzymes (CHIT 102, CHIT 64, CHIT 72 and CHIT 73) belonging to the β-N-acetylglucsaminidase group are reported (11) and two genes have been cloned (6, 13).

Trichoderma atroviride is a unique species isolated from Alaska. *T. atroviride* is a cold tolerant strain and parasitizes a wide range of plant pathogenic fungi (14). Previous studies have shown that *T. atroviride* produced chitinases, glucanases and proteinases (3). This chapter characterizes the 73 kDa β-N-acetylglucosaminidase gene from three *T. atroviride* biotypes.

Materials and Methods

Genomic DNA extraction. DNA was extracted using the method of Carlson *et al* (1991) (2) where actively growing white mycelia of *T. atroviride* was scraped from potato

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dextrose agar (PDA) plates resuspend in 1.5 ml lysis buffer (100 mM Tris, pH 9.5, 20 mM EDTA, 1.4 M NaCl, 2 % CTAB and 1% PEG 8000 or 6000) and transferred into 1.5 ml microcentrifuge tubes. The tubes were incubated in a 74 °C water bath for 1 hour with inverted mixing every 10 minutes and then centrifuged 10 minutes at 14000 rpm. The supernatant was transferred into new tubes and 3 × 1:1 Phenol:chloroform was added. After centrifugation for 10 minutes at 14000 rpm, the aqueous phase was recovered. Then 2 × ethanol was added and this mixture was incubated at -20 °C for one hour before centrifuging for 10 minutes at 14000 rpm. The pellets were washed with 70 % ethanol and dried in A Speed Vac. Dried pellet were dissolved in 100 µl TE buffer (20 mM Tris, 10mM EDTA, pH 8.0).

Primer selection, PCR amplification and sequencing. The primers for amplification of 73 kDa β-N-acetylglucosaminidase were selected according to the published sequence of 73 kDa β-N-acetylglucosaminidase gene from *T. harzianum* P1 in GeneBank (GeneBank acc. NO. S83231) (13). Primers were obtained from MWG-Biotech (High Point, NC). A PCR amplification kit (Takara Bio, Shiga, Japan) was used to amplify the 73 kDa β-N-acetylglucosaminidase gene by using primers nagf1 and nagr1 (Table 6.1). Reaction mixtures contained 78.5 µl of sterile distilled water; 10 µl of 10 × Ex Taq™ buffer; 8 µl of 2.5 mM each of dNTP, 0.1 µm primer, 1 µl of genomic DNA template, and 2.5 U of Takara Ex Taq™. Reactions were performed in tubes using DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA) with the following program: one cycle at 96 °C for 5 minute; three cycles at 96 °C for 1 minute, 40 °C for two minutes, and 72 °C for three minutes; 35 cycles at 96 °C for 1 minute, 50 °C for 2 minutes, and 72 °C for 3 minutes;

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and one cycle at 72 °C for 10 minutes. The PCR products were analyzed by electrophoresis on a 1.2 % agarose gel. The PCR products were purified using Quantum Prep® Freeze N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA). Thirteen primers in forward and reverse orientations (Table 6.1) were used to sequence the purified products using an ABI PRISM® Big Dye™ Terminator v3.0 Cycle Sequencing Kit and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). Sequence comparison and alignment were completed with Clustal W analysis (EMBL-EBI, Cambridge, UK) (9) and Multalin (4).

Results

There were two distinct bands on agarose gel (Figure 6.1). One was about 2.7 kb and the other about 700 base pairs. The 2.7 kb PCR product was selected, purified and sequenced. A Total of 2416, 2418 and 2564 base pairs were obtained from 603, 453 and 861, respectively. Cluster alignment showed that the sequence of cold tolerant *T. atroviride* biotypes were different from the sequence of *T. harzianum* P1. The mutants of *T. atroviride,* biotype 603 and 453 belonged to one group and differed from the wild biotype 861 (Figure 6.3). There were 11 unique base pairs for *T. atroviride* biotypes when compared with the published β -N-acetylglucosaminidase gene from GeneBank (Figure 6.2). The number of base pair insertions was 4, 5 and 5 and single base pair change was 13, 13, and 9 for 603, 453 and 861, respectively (Figure 6.2).

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Discussion

The 73 kDa β-N-acetylglucosaminidase genes were amplified from three *T. atroviride* biotypes 861, 603 and 453. That means the cold tolerant T. atroviride contained the genes. However, the study showed *B. cinerea*, *P. capsici* and *R. solani* did not induce the production of 73 kDa β-N-acetylglucosaminidase from *T. atroviride*. Only *S. sclerotiorum* induced the production of 73 kDa β -N-acetylglucosaminidase (data not shown). That indicated the 73 kDa β -N-acetylglucosaminidase was a plant pathogen inducible enzyme. The autoclaved mycelia of *B. cinerea*, *P. capsici*, *R. solani*, and *S. sclerotiorum* also did not induce the production of 73 kDa β -N-acetylglucosaminidase. Thus proteins produced by *S. sclerotiorum* induce the production of 73 kDa β -N-acetylglucosaminidase. Further studies are needed to determine what kind of proteins produced by *S. sclerotiorum* play a role in inducing the 73 kDa β –N-acetylglucosaminidase production.

Another cold tolerant *T. atroviride* biotype, 901, also produced 73 kDa β -N-acetylglucosaminidase (data not shown), But it was difficult to get the PCR product because the 2.7 kb bank was too weak to get enough DNA to sequence. That may mean the concentration of the isolated DNA was lower than those from other *T. atroviride* biotypes under the isolation condition used.

The sequence of different cold tolerant *T. atroviride* biotypes were unique biotypes and differed from the published sequence from GeneBank. It can be used to identify the species and isolates of *Trichoderma spp.*

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Table 6.1 Primers used in PCR and sequencing for 73 kDa β-N-acetyl-D-glucosaminidase.

Primers	Sequence
Nagf1	5'-ACATTAACGTAACGTTGCGCG-3'
Nagf2	5'-GACAGCGTTCTCTTCATTGACGA-3'
Nagf3	5'-TTACCAGACGGTCGCCGACGATAT-3'
Nagf4	5'-GATTTCGTTGATAAACTGTTTGACGAC-3'
Nagf5	5'-GCGACACTGTCGTTCAATCGT-3'
Nagr1	5'-TTGAATCAAAACAAATCGAATACC-3'
Nagr2	5'-AGTCACTCTCACAGCCTCGTCAAT-3'
Nagr3	5'-GTCAATGGTTCTCTTGATATCGGC-3'
Nagr4	5'-AATCGTAAACCTTGGAGTCGTTGA-3'
Nagr5	5'-CAGCCACGATTGAACGACAGT-3'
Nagr6	5'-ACACTCCATCCTAGACTTCTTATGCG-3'
Glufor2	5'-AGTGCCAAGACCTCTACCGGTATC-3'
Glurev1comp2	5'-GATACCGGTAGAGGTCTTGGCACT-3'

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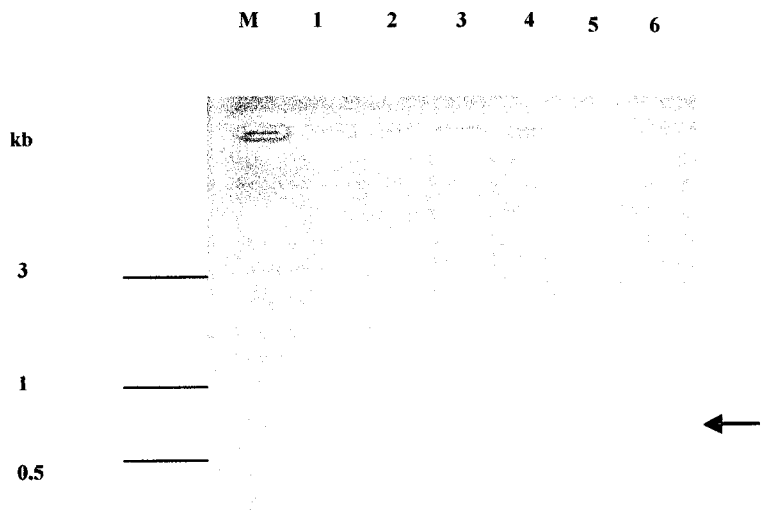

Figure 6.1. Agarose gel electrophoresis PCR product of *T. atroviride* biotypes. The arrow shows the 2.7 kb PCR product. Lane M: DNA marker (0.5-12 kb); lane1: *T. atroviride* biotypes 453; lane 2: *T. atroviride* biotype 603; lane 3: *T. atroviride* biotype 861; lane 4 and 6: *T. atroviride* biotype 901; lane 5: PCR control.

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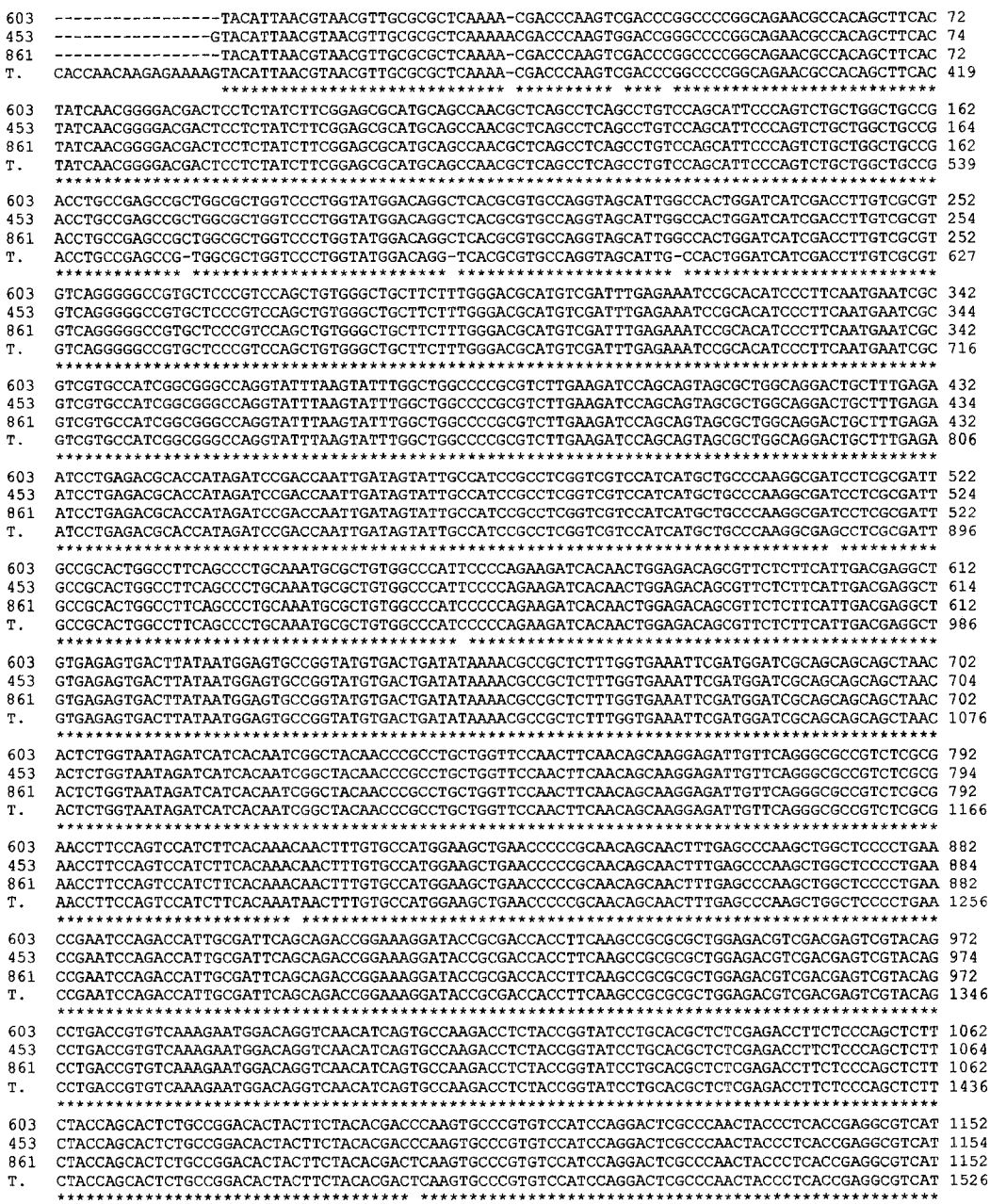

Figure 6. 2. Sequence comparison. Multalin sequence comparison of N-acetyl-D-glucosaminidase genes of three *T. atroviride* biotypes with the published *nag1* gene sequence of *T. harzianum* P1. 861=*T. atroviride* biotypes 861, 453=*T. atroviride* biotypes 453, 603=*T. atroviride* biotype 603 and P1=*T. harzianum* P1.

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603  GCTTGATCTTGCCCGTACTTACCAGACGGTCGCCGATATCAAGAGAACCATTGACGCCATGTCCTGGAACAAGCTCAACCGTCTGCACTT 1242
453  GCTTGATCTTGCCCGTACTTACCAGACGGTCGCCGATATCAAGAGAACCATTGACGCCATGTCCTGGAACAAGCTCAACCGTCTGCACTT 1244
861  GCTTGATCTTGCCCGTACTTACCAGACGGTCGCCGATATCAAGAGAACCATTGACGCCATGTCCTGGAACAAGCTCAACCGTCTGCACTT 1242
T.   GCTTGATCTTGCCCGTACTTACCAGACGGTCGCCGATATCAAGAGAACCATTGACGCCATGTCCTGGAACAAGCTCAACCGTCTGCACTT 1616
     ********************************************************************************************

603  GCACATCACCGACTCCCAGTCGTGGCCTCTGGTGATCCCTTCACTGCCTAAGCTGTCTCAGGAGGGTGCCTACCACCCAAGCCTGGTCTA 1332
453  GCACATCACCGACTCCCAGTCGTGGCCTCTGGTGATCCCTTCACTGCCTAAGCTGTCTCAGGAGGGTGCCTACCACCCAAGCCTGGTCTA 1334
861  GCACATCACCGACTCCCAGTCGTGGCCTCTGGTGATCCCTTCACTGCCTAAGCTGTCTCAGGAGGGTGCCTACCACCCAAGCCTGGTCTA 1332
T.   GCACATCACCGACTCCCAGTCGTGGCCTCTGGTGATCCCTTCACTGCCTAAGCTGTCTCAGGAGGGTGCCTACCACCCAAGCCTGGTCTA 1706
     ********************************************************************************************

603  TTCTCCCGCCGATCTTGCAGGCATTTTCCAATACGGCATTGACCGCGGTGTTGAGGTCATCACCGAGATCGACATGCCCGGTCACATTGG 1422
453  TTCTCCCGCCGATCTTGCAGGCATTTTCCAATACGGCATTGACCGCGGTGTTGAGGTCATCACCGAGATCGACATGCCCGGTCACATTGG 1424
861  TTCTCCCGCCGATCTTGCAGGCATTTTCCAATACGGCATTGACCGCGGTGTTGAGGTCATCACCGAGATCGACATGCCCGGTCACATTGG 1422
T.   TTCTCCCGCCGATCTTGCAGGCATTTTCCAATACGGCATTGACCGCGGTGTTGAGGTCATCACCGAGATCGACATGCCCGGTCACATTGG 1796
     ********************************************************************************************

603  CGTTGTCGAGCTTGCTTACAGCGACCTCATTGTGGCCTACCAAGAGATGCCCTACCAATACTACTGCGCCGAGCCGCCATGTGGTGCCTT 1512
453  CGTTGTCGAGCTTGCTTACAGCGACCTCATTGTGGCCTACCAAGAGATGCCCTACCAATACTACTGCGCCGAGCCGCCATGTGGTGCCTT 1514
861  CGTTGTCGAGCTTGCTTACAGCGACCTCATTGTGGCCTACCAAGAGATGCCCTACCAATACTACTGCGCCGAGCCGCCATGTGGTGCCTT 1512
T.   CGTTGTCGAGCTTGCTTACAGCGACCTCATTGTGGCCTACCAAGAGATGCCCTACCAATACTACTGCGCCGAGCCGCCATGTGGTGCCTT 1886
     ********************************************************************************************

603  CTCGCTCAACGACTCCAAGGTTTACGATTTCGTTGATAAACTGTTTGACGACCTTTTGCCCCGTGTCACGCCTTACAGCTCCTACTTCCA 1602
453  CTCGCTCAACGACTCCAAGGTTTACGATTTCGTTGATAAACTGTTTGACGACCTTTTGCCCCGTGTCACGCCTTACAGCTCCTACTTCCA 1604
861  CTCGCTCAACGACTCCAAGGTTTACGATTTCGTTGATAAACTGTTTGACGACCTTTTGCCCCGTGTCACGCCTTACAGCTCCTACTTCCA 1602
T.   CTCGTTCAACGACTCCAAGGTTTACGATTTCGTTGATAAACTGTTTGACGACCTTTTGCCCCGTGTCACGCCTTACAGCTCCTACTTCCA 1976
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603  CACCGGTGGTGATGAGCTCAACGCCAACGACTCCATGATCGACCCCCGTCTCAAGTCCAACTCGAGCGATGTCCTACAGCCTCTGCTGCA 1692
453  CACCGGTGGTGATGAGCTCAACGCCAACGACTCCATGATCGACCCCCGTCTCAAGTCCAACTCGAGCGATGTCCTACAGCCTCTGCTGCA 1694
861  CACCGGTGGTGATGAGCTCAACGCCAACGACTCCATGATCGACCCCCGTCTCAAGTCCAACTCGAGCGATGTCCTACAGCCTCTGCTGCA 1692
T.   CACCGGTGGTGATGAGCTCAACGCCAACGACTCCATGATCGACCCCCGTTATAAGTCCAACTCGAGCGATGTCCTACAGCCTCTGCTGCA 2066
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603  AAAGTTCATCTCTCATGCTCACTCCAAGATCCGCGCCCAGGGTCTTTCACCGCTTGTCTGGGAGGAGATGGTCACCACCTGGAACCTGAC 1782
453  AAAGTTCATCTCTCATGCTCACTCCAAGATCCGCGCCCAGGGTCTTTCACCGCTTGTCTGGGAGGAGATGGTCACCACCTGGAACCTGAC 1784
861  AAAGTTCATCTCTCATGCTCACTCCAAGATCCGCGCCCAGGGTCTTTCACCGCTTGTCTGGGAGGAGATGGTCACCACCTGGAACCTGAC 1782
T.   AAAGTTCATCTCTCATGCTCACTCCAAGATCCGCGCCCAGGGTCTTTCACCGCTTGTCTGTGAGGAGATGGTCACCACCTGGAACCTGAC 2156
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603  TCTTGGCAGCGACACTGTCGTTCAATCGTGGCTGGGTGGTGATGCCGTCAAGAACTTGGCCGAGTCTGGCTACAAGGTCATTGACACCGA 1872
453  TCTTGGCAGCGACACTGTCGTTCAATCGTGGCTGGGTGGTGATGCCGTCAAGAACTTGGCCGAGTCTGGCTACAAGGTCATTGACACCGA 1874
861  TCTTGGCAGCGACACTGTCGTTCAATCGTGGCTGGGTGGTGATGCCGTCAAGAACTTGGCCGAGTCTGGCTACAAGGTCATTGACACCGA 1872
T.   TCTTGGCAGCGACACTGTCGTTCAATCGTGGCTGGGTGGTGATGCCGTCAAGAACTTGGCCGAGTCTGGCTACAAGGTCATTGACACCGA 2246
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603  CTACAACTTTTACGTGAGTTTGATGATTTTCTGAACAATCCGATGTAGCTGGATATTTGCTGACGAAACCATACAGTACCTGGACTGCGG 1962
453  CTACAACTTTTACGTGAGTTTGATGATTTTCTGAACAATCCGATGTAGCTGGATATTTGCTGACGAAACCATACAGTACCTGGACTGCGG 1964
861  CTACAACTTTTACGTGAGTTTGATGATTTTCTGAACAATCCGATGTAGCTGGATATTTGCTGACGAAACCATACAGTACCTGGACTGCGG 1962
T.   CTACAACTTTTACGTGAGTTTGATGATTTTCTGAACAATCCGATGTAGCTGGATATTTGCTGACGAAACCATACAGTACCTGGACTGCGG 2336
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861  GCTCATCTACTCTCACGACCCTGCCAAGGGCGTCTCCAAGGCGAATGCCAAGAACGTCTTGGGAGGAGAGCTTGCCATCTGGAGTGAGAT 2142
T.   GCTCATCTACTCTCACGACCCTGCCAAGGGCGTCTCCAAGGCGAATGCCAAGAACGTCTTGGGAGGAGAGCTTGCCATCTGGAGTGAGAT 2516
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453  GATTGACGGCAGCAACATGGACAACATCATCTGGCCCCGTGGCAGTGCCGCCGGCGAGGTCTGGTGGTCCGGCAACGTTGATACCGCGAC 2234
861  GATTGACGGCAGCAACATGGACAACATCATCTGGCCCCGTGGCAGTGCCGCCGGCGAGGTCTGGTGGTCTGGCAACGTTGATACCGCGAC 2232
T.   GATTGACGGCAGCAACATGGACAACATCATCTGGCCCCGTGGCAGTGCCGCCGGCGAGGTCTGGTGGTCTGGCAACGTTGATACCGCGAC 2606
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603  CGGCCAGAACCGCAGCCAGCTCGAGGTGACGCCTCGCTTGAACGAGTTCCGTGAGCGTATGCTTGCGCGTGGTGTCAATGCCATGCCCAT 2322
453  CGGCCAGAACCGCAGCCAGCTCGAGGTGACGCCTCGCTTGAACGAGTTCCGTGAGCGTATGCTTGCGCGTGGTGTCAATGCCATGCCCAT 2324
861  CGGCCAGAACCGCAGCCAGCTCGAGGTGACGCCTCGCTTGAACGAGTTCCGTGAGCGTATGCTTGCGCGTGGTGTCAACGCCATGCCCAT 2322
T.   CGGCCAGAACCGCAGCCAGCTCGAGGTGACGCCTCGCTTGAACGAGTTCCGTGAGCGTATGCTTGCGCGTGGTGTCAACGCCATGCCCAT 2696
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603  CCAGATGACTTACTGCACGCAGCTCAACGCTACGGCTTGCACGCTGTTCGCATAAGAAGTCTAGGGTAGAGTGTTTCTCAATTGGAGATT 2412
453  CCAGATGACTTACTGCACGCAGCTCAACGCTACGGCTTGCACCTTGTTCGCATAAGAAGTCTAGGGTAGAGTGTTTCTCAATTGGAGATT 2414
861  CCAGATGACTTACTGCACGCAGCTCAACGCTACGGCTTGCACCTTGTTCGCATAAGAAGTCTAGGATGGAGTGTTTCTCAATTGGAGATT 2412
T.   CCAGATGACTTACTGCACGCAGCTCAACGCTACGGCTTGCACCTTGTTCGCATAAGAAGTCTAGGATGGAGTGTT-CTCAATTGGAGATT 2786
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603  GGTT------------------------------------------------------------------------------------- 2416
453  GGTT------------------------------------------------------------------------------------- 2418
861  GGTTTTTGTTTTATTAATTAGCATTGTTTAATGTACAAAGGTTAAAACAGGTTTATGGTTTATGAGAAGTTTCGGGGCAATGGCTTGGGG 2502
T.   GGTTTTTGTTT-ATTAATTAGCATTGGTTAATGTACAAAGGTTAAAACAGGTTTATGGTTTATGAGAAGTTTCGGGGCAATGGCTTGGGG 2874
     ****
603  -------------------------------------------------------------------------------------------
453  -------------------------------------------------------------------------------------------
861  CAATATCTGTTCTATAACTGTATATATATAATTCATTAATACCCTGAACTTTCAACTTGATC---------------------------- 2564
T.   CAATATCTGGTCTATAACTGTATATATATAATTCATTAATACCCTGAACTTTCAACTTGATCTTTTTTTGTGCATATCATCGGTTGTGAT 2964
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Figure 6.2 cont'd.

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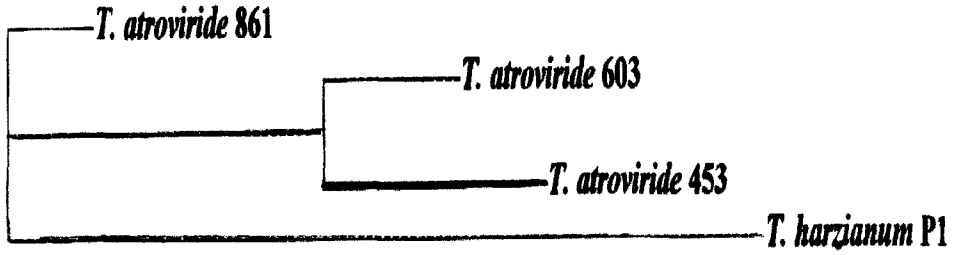

Figure 6.3. Phylogenetic tree. Phylogenetic comparison of the N-acetyl-D-glucosaminidase gene *nag1* sequence among three *T. atroviride* biotypes and *T. harzianum* P1.

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CHAPTER 7: β-1,6-GLUCANASE ISOENZYME PROFILES OF A COLD TOLERANT *TRICHODERMA ATROVIRIDE*

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Abstract

β-1,6-glucan is component of the cell wall of plant pathogens and β-1,6-glucanase produced by *Trichoderma* may play a role in its mycoparasitism of other fungi. β-1,6-glucanases produced by cold tolerant *T. atroviride* were influenced by carbon source and pH. *T. atroviride* produced multiple β-1,6-glucanases when it was grown with glucose, glycol chitosan, pustulan, and autoclaved mycelia of several plant pathogens (*Botrytis cinerea, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum*) as the sole carbon sources. Molecular weight of the various β-1,6-glucanases detected by SDS-PAGE ranged from 51 to 200 kDa. β-1,6-glucanase was detected from pH 2.0 to pH 7.0 and specific activity peaked at pH 4.0. β-1,6-glucanase activity was highest on the second day after *T. atroviride* was transferred from repression to induction conditions, decreased the third day, and then increased again the fourth and fifth day to the-second-day's level. Understanding β-1,6-glucanase profiles produced by *T. atroviride* helps understanding mechanisms of phytopathogen biocontrol.

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Introduction

Chitin and β-1,3-glucans are the two main components of the cell walls of plant pathogenic fungi (exception one Oomycetes) (5). Extensive studies on chitinases and β-1,3-glucanases involved in mycoparasitism by *Trichoderma* species biocontrol have been reported (2,6,7,12,14,15,16,24). Proteins and β-1,6-glucans are also components of fungal cell walls although they are minor polymers (1,5). Proteinases and β-1, 6-glucanase produced by *Trichoderma* species may play a role in biocontrol by *Trichoderma* biocontrol. Involvement of proteinase in biocontrol by *Trichoderma* was shown by improving the biocontrol activity after overproduction of proteinase (Prb1) (11), hydrolyzing *Crinipellis perniciosa* cell wall (9) and serving as an elicitor of plant defense compound (13). Purified β-1,6-glucanases (BGN16.1 and BGN16.2) of *Trichoderma harzianum* were showed hydrolytic activity and inhibited the growth of fungi when combined with β-1,3-glucanase and chitinase (7,8). Information about expression of β-1,6-glucnases in *Trichoderma* are more limited than with other hydrolytic enzymes (7,8,23).

Trichoderma atroviride, a cold tolerant biocontrol agent, parasitizes a wide range of plant pathogenic fungi (26). Mycoparasitism is one of the mechanisms involved in biocontrol by *T. atroviride* and *T. atroviride* is reported to produce cell wall-degrading enzymes (4,26). In this chapter, I report the β-1,6-glucanase profiles of *T. atroviride* with different carbon sources and pHs over time course of β-1,6-glucanase production.

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Materials and Methods

Chemicals and reagents. Pustulan from *Umbilicaria papullosa* (Calbiochem, La Jolla, CA) was used as the β-1,6-glucanase substrate standard. Disposable semi-micro (1.5 ml) cuvettes, 12 % Longlife Gel (Gradipore Ltd., Frenchs Forest, Australia) purchased from (VWR, Bristol, CT). SDS-PAGE protein standard (Bio-Rad, Hercules, CA), Bradford Reagent, glycol chitosan and Czapek-Dox broth (Sigma, St. Louis. MO), and baking yeast (Fleishmann's band) were used in this study.

Plant pathogenic fungal mycelia preparation. The plant pathogenic fungi selected to induce production of β-1,6-glucanase (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*). They were grown in potato dextrose broth for 10 days before filtering, air drying and grinding the mycelium.

Growth condition for β-1,6-glucanase induction with different carbon sources. *Trichoderma atroviride* biotype 901 was used in this experiment. Conidia of *T. atroviride* from 10-day potato dextrose agar were added to Czapek medium supplemented with 10 % (wt/vol) glucose for a final concentration of 10^6 spores/ml and shaken for 4 days at 140 rpm at room temperature (repression conditions) (7). Mycelia were washed with 2 % (wt/vol) $MgCl_2$ (7) and transferred to each media containing 0.5 % autoclaved mycelia of plant pathogens or other carbon sources (pustulan, yeast, glycol chitosan and glucose). Mycelium of *T. atroviride* was filtered through paper (VWR, Bristol, CT) after a 4-day incubation at 140 rpm on a rotary shaker at room temperature. The filtrate was concentrated under vacuum.

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The effect of pH and time on the production of β-1,6-glucanase. Citric acid and sodium phosphate were adjusted to make 50 mM pH 2.0 to pH 8.0 buffers. Sodium borate and sodium hydroxide were used to make 50 mM pH 9.0 to pH 10.0 buffers. β-1,6-glucanase induction followed the procedures described by de la Cruz (7). Mycelia from repression conditions were transferred to media with 0.5 % autoclaved mycelia of *R. solani* at the different pH and shaken for four days at room temperature and 140 rpm. Mycelia were filtered through filter paper and vacuum concentrated. A similar study at pH 5, mycelium was collected after 1, 2, 3, 4, or 5 days. The culture was filtered and vacuum concentrated.

β-1,6-glucanase and protein assay. Pustulan was used as the β-1,6-glucanase substrate (7,19,22). The 400 µl reaction mixture, containing 80 µl of sample solution, 160 µl of 50 mM pH 5.0 acetate buffer and 160 µl of 0.5 % laminarin in 50 mM pH 5.0 acetate buffer, was incubated at 37 °C for 30 minutes. Then one ml of solution A (40.0 g/L Na_2CO_3, 16.0 g/L glycine and 0.45 g/L $CuSO_4$) and one ml of solution B (0.12 g/L neocuproine hydrochloride) were added to the reaction mixture and autoclaved for 10 minutes to develop the color (27). Other samples were boiled for 5 minutes as controls. Absorbance was measured spectrophotometicelly at 440 nm. One unit of β-1,6-glucanase was defined as one milliliter of enzyme producing one micromole glucose per minute. Bradford reagent (30 %) was used to determine protein concentration (3). Fifty microliters of sample were added to 2 ml of 30 % Bradford reagent, the mixture was incubated for 30 minutes at room temperature and absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as a reference protein.

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Enzymes separated by SDS-PAGE were renatured with a casein-EDTA wash (18) and through an agarose replica gel to detect β-1,6-glucanase activity (23).

Statistical analysis. All experiments were designed with three replications and repeated twice. The data were analyzed using ANOVA and the GLM procedure of SAS with lsmeans (SAS Institute Inc. 1999) (20).

Results

The effect of carbon sources on the production of β-1,6-glucanase. Carbon source greatly influenced the production and size of β-1,6-glucanase. Multiple β-1,6-glucanases were produced by *T. atroviride* with different carbon sources. β-1,6-glucanases were detectable when glucose, glycol chitosan, pustulan, and the autoclaved mycelia of plant pathogens (*B. cinerea*, *P. capsici*, *R. solani* and *S. sclerotiorum*) were used as carbon sources (Figure 7.1). No β-1,6-glucanase was detected when yeast was used as the sole carbon source (Figures 7.1 and 7.2). Gel clarified that the autoclaved mycelia of *B. cinerea* and *R. solani* induced *T. atroviride* to produce at least two different sizes of β-1,6-glucanase (one was about 66 kDa and the other about 200 kDa) (Figure 7.2). One 51 kDa β-1,6-glucanase was detected when pustulan and the autoclaved mycelia of *P. capsici* were used as the carbon sources, a 200 kDa β-1,6-glucanase band was detected when glycol chitosan used as the sole carbon source (Figure 7.2). No β-1,6-glucanase band was detected when the autoclaved mycelia of *S. sclerotiorum* and glucose were used as the sole carbon source (Figure 7.2).

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The effect of pH and time on the production of β-1,6-glucanase. β-1,6-glucanase induction was affected by pH (Figure 7.3). The β-1,6-glucanase was detected when *T. atroviride* was grown at pH from 2 to 7; however, there was no β-1,6-glucanase activity detected when *T. atroviride* was grown at pH 8.0, 9.0 and 10.0. The specific activity of β-1,6-glucanase peaked at pH 4.

β-1,6-glucanase activity was detected after one days growth of *T. atroviride*. The specific activity of β-1,6-glucanase peaked the second day, decreased the third day, and increased again the fourth day. There were no significant differences in specific activity between the second, fourth and fifth day (Figure 7.4).

Discussion

T. harzianum produced more β-1,6-glucanase with chitin, pustulan, nigeran, and the cell walls than in glucose, fructose, glycerol or laminarin amended media (6). *T. atroviride* produced β-1,6-glucanase when it was grown with glucose, glycol chitosan, pustulan and autoclaved fungal mycelia. In contract to *T. harzianum*, there were no significant differences with *T. atroviride* among the different substrates tested (except yeast) although the autoclaved mycelia of *B. cinerea* and *R. solani* induced more β-1,6-glucanse than the other substrates (Figure 7.1). The expression of β-1,6-glucanase isozymes was influenced by carbon source and probably reflects differences in the carbon source structure and cell wall component differences (1, 5).

Cell wall degrading enzymes produced by *Trichoderma* are more complex than initially thought. At lease 10 different molecular weight chitinase isozymes and seven β-1,3-

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glucanase isozymes have been reported from *T. harzianum* (17, 25). Two β-1,6-glucanase isozymes (43 kDa and 51 kDa) have been purified from *T. harzianum* (7,8); however, the presence of more than two β-1,6-glucanase isozymes of *T. harzianum* has been suggested (23). At least three different molecular weight β-1,6-glucanase isozymes were detected from *T. atroviride* (Figure 7.2). The 51 kDa β-1,6-glucanase may be the same as reported from *T. harzianum* (8). The molecular weights of the other two β-1,6-glucanase isozymes of *T. atroviride* are larger than β-1,6-glucanase reported from *T. harzianum*. Additional characterization of β-1,6-glucanase isozymes are needed to fully understand the role they play in biocontrol by *T. atroviride*.

β-1,6-glucan is component of yeast (*Saccharomyces cerevisiae*) (21) and autoclaved cell wall of yeast induced *T. harzianum* to produce more β-1,6-glucanase than glucose (6). In contrast, β-1,6-glucanase activity was not detected when *T. atroviride* was grown with autoclaved yeast (Figures 7.1 and 7.2). This may be explained by the presence of easily digested substrate in the formulation that *T. atroviride* prefers over β-1,6-glucan. Low nutrient condition may be needed for β-1,6-glucanase production as supported for endochitinase CHIT42 and N-acetyl-D-glucosaminidase NAG1 (10).

It is clear from this research that *T. atroviride* can produce at least three different molecular weights β-1,6-glucanase isozymes and that isozyme production influenced by the carbon source. pH also influenced production of β-1,6-glucanase. The specific activity of β-1,6-glucanase peaked the second day of incubation. Further studies to characterize the two new β-1,6-glucanase isozymes will help define their role the role in biocontrol by *T. atroviride*.

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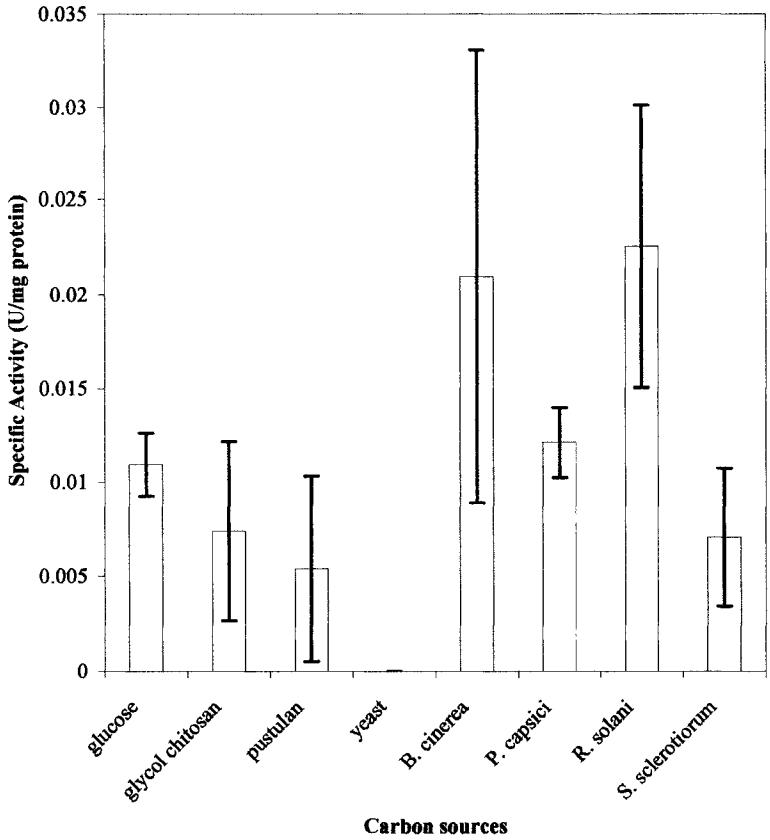

Figure 7.1. The effect of carbon source on the production of β-1,6-glucanase. The values are the average of three replications and the error bar is the standard error.

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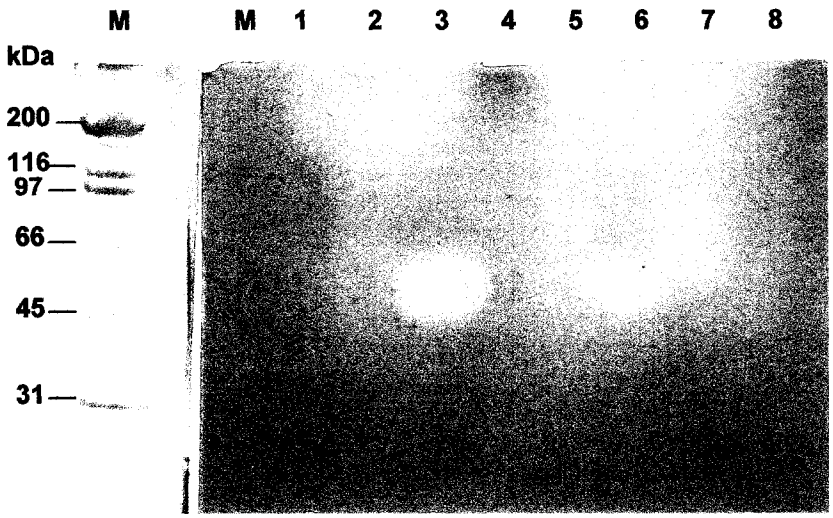

Figure 7.2. β-1,6-glucanase isozymes. Activity staining of β-1,6-glucanase isozyme produced by T. atroviride under different carbon sources after SDS-PAGE in a replica agarose gel. Each lane was 1.5 μg total proteins. M=protein molecular weights marker; 1, glucose; 2, glycol chitosan; 3, pustulan; 4, yeast; 5, the autoclaved mycelia of *B. cinerea*; 6, the autoclaved mycelia of *P. capsici*; 7, the autoclaved mycelia of *R. solani*; 8, the autoclaved mycelia of *S. sclerotiorum*.

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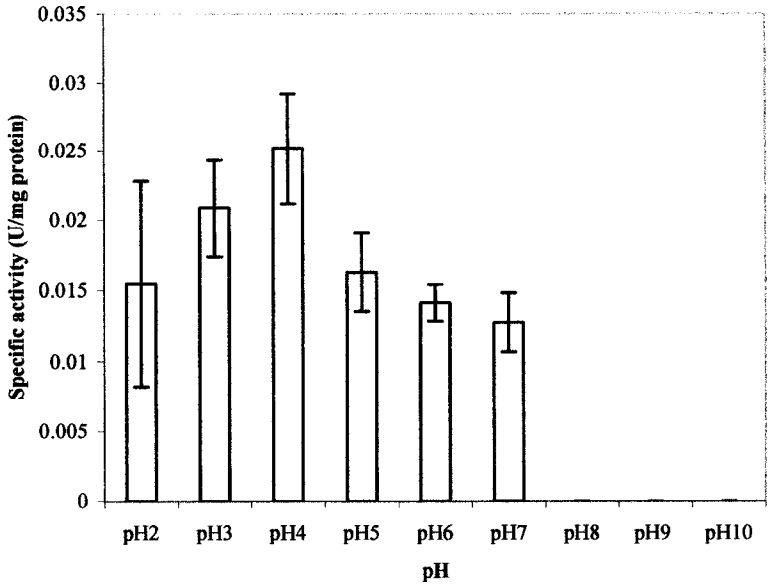

Figure 7.3. The effect of pH on the production of β-1,6-glucanase. The values are the average of three replications and the error bar is the standard error.

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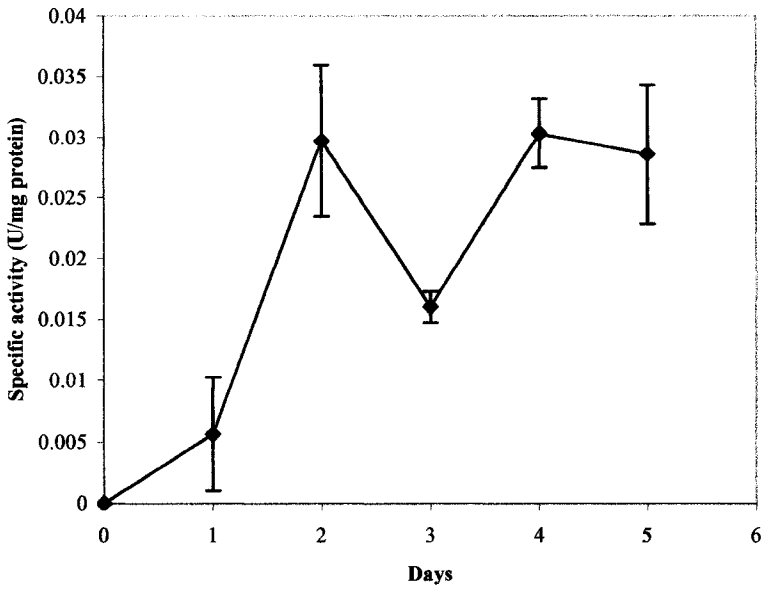

Figure 7.4. The time-course of β-1,6-glucanase produced by *T. atroviride*. The values are the average of three replications and the error bar is the standard error.

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CHAPTER 8: THE REGULATION OF β-N-ACETYL-D-GLUCOSAMINIDASE IN *TRICHODERMA ATROVIRIDE BY* PLANT PATHOGENS

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Abstract

Trichoderma atroviride is a cold tolerant fungus that parasitizes a wide range of plant pathogenic fungi. β-N-acetyl-D-glucosaminidase is one of the important enzymes involved in biocontrol by *T. atroviride*. Two β -N-acetyl-D-glucosaminidases were produced by *T. atroviride* when it was grown with plant pathogenic fungi (*Botrytis cinerea, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum*) and their autoclaved mycelia. SDS-PAGE and native PAGE showed that these two β-N-acetyl-D-glucosaminidases were different. One is about 93 kDa and the other is about 73 kDa. The 73 kDa β-N-acetyl-D-glucosaminidase was produced only when *T. atroviride* was grown with *S. sclerotiorum*. *T. atroviride* produced the 93 kDa β-N-acetyl-D-glucosaminidase when it was grown with other plant pathogenic fungi and their autoclaved mycelia. Culture filtrates of *S. sclerotiorum* regulated production of the 73 kDa β-N-acetyl-D-glucosaminidase while culture filtrates of *B. cinerea* had no effect on its expression. Metabolites from *S. sclerotiorum* culture filtrate were released into the media and regulated expression of the 73 kDa β-N-acetyl-D-glucosaminidase. Expression of the 73 kDa β-N-acetyl-D-glucosaminidase occurred before physical contact of *T. atroviride* with *S. sclerotiorum*.

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Introduction

Chitin, the β -1, 4-linked homopolymer of N-acetyl-D-glucosamine, is a major component of the cell walls of fungi (except the groups belong to Oomycetes) (5). *Trichoderma* and *Gliocladium* are well-known chitinase producers and there is a correlation between chitinase production and their biocontrol ability (10). There are three major chitinase isoenzyms, i.e. β-N-acetyl-D-glucosaminidase (NAGase) (EC 3.2.1.30) (β-N-acetylhexosaminidase (EC 3.2.1.52), chitin 1, 4-chitobiosidase (chitobiosidase or exochitinase) and endochitinase (EC 3.2.1.14), produced by *Trichoderma* species (8). The Nag1 N-acetyl-D-glucosaminidase is very important for biocontrol by *T. atroviride* (2). Thirty percent of the biocontrol of *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was lost by *nag1*-disruption in *T. atroviride* and *nag1*-disruption also blocked the production of other chitinases (2). Research on the regulation of N-acetyl-glucosaminidase expression can provide a better understanding of mechanisms involved in biocontrol by *Trichoderma.*

The production of NAGases is affected by the host and their expression is very specific and finely tuned (7). Fungal cell walls, autoclaved mycelia of plant pathogenic fungi, chitin, colloidal chitin and chitin degradation products trigger the production of N-acetyl-D-glucosaminidase (6,7,12,14,15). An underglycosylated form of N-acetyl-D-glucosaminidase was produced by *T. harzianum* when tunicamycin (an inhibitor of protein N- glycosylation) was in the media (17). N-acetyl-D-glucosaminidase production was repressed by cAMP (16) and deoxynivalenol produced by *Fusarium culmorum* and *F. graminearum* (11). Reports on timing of the expression of NAGases have been

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controversial. Some reports showed that 73 kDa NAGase was expressed after *Trichoderma* made physical contact (7,20) while other reports concluded that expression of the 73 kDa NAGase was contact independent and triggered by a diffusible factor (4,15).

Trichoderma atroviride 901 is a cold tolerant strain that parasitizes plant pathogenic fungi (19) and produces cell wall degrading enzymes (chitinases, glucanases and proteinase) when grown with autoclaved mycelia of plant pathogenic fungi (3). This chapter reports the regulation of *T. atroviride* NAGase expression by plant pathogenic fungi, the timing of NAGase expression, and the effect of extracellular metabolites from *S. sclerotiorum* on the expression of NAGases.

Materials and Methods

Fungal strains and media. Plant pathogenic fungi used were *Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. *T. atroviride* 901 was used through the entire experiment. The media used for enzyme production is minimal media (0.2% asparagine, 0.2% $NaNO_3$, 0.05% $MgSO_4$ $7H_20$, 0.05 % KCl, and 0.1% KH_2PO_4) (18). Plant pathogen mycelia were harvested from potato dextrose broth cultures (Difco, Spark, MD).

The effect of plant pathogenic fungi on the expression of NAGase. Conidia of *T. atroviride* from a 10-day potato dextrose agar (PDA) culture were inoculated into 100 ml of minimal media supplemented with 0.5 % autoclaved mycelia of plant pathogens. Two 0.06 cm^2 plugs of the plant pathogens from PDA plate were inoculated with 10^7 spores of

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T. atroviride at the same time. The fungi were grown at room temperature in shake cultures at 140 rpm for four days before filtering the mycelia on a 0.45 μm Millipore® filter (Bedford, MA) and concentrating the supernatant to 1.6 ml under vacuum at room temperature. Each treatment had three replications and the whole experiment was repeated once.

The effect of pathogen filtrates on the expression of NAGase. Two 0.06 cm^2 plugs of 10-day *B. cinerea* and *S. sclerotiorum* from a potato dextrose agar plate were added to 100 mL of minimal media and inoculated at room temperature on a rotary shaker at 140 rpm for one day, two days or three days. The mycelia of each pathogen were filtrated on a 0.45 μm Millipore® filter before filtering on a 0.20 μm sterile syringe filter (Corning Incorporated, Corning, NY) to remove all mycelia or conidia of the plant pathogenic fungi. The filtrates were inoculated with *T. atroviride* spores, grown at room temperature, filtered and concentrated as previously described. Each treatment had three replications and the whole experiment was repeated once.

The effect of *S. sclerotiorum* mycelia and extract from mycelia on the expression of NAGase. *S. sclerotiorum* was grown in minimal media at room temperature in shake culture at 140 rpm for ten days. Mycelia were filtrated on a 0.45 μm Millipore® filter, ground aseptically, added to 100 mL of minimal media and filtrated through a 0.45 μm Millipore® filter. The filtrates were again filtered through a 0.20 μm sterile syringe filter, inoculated with *T. atroviride* spores, grown at room temperature for four days, filtered and concentrated as above. The mycelium of *S. sclerotiorum* was washed three times with sterile water and transferred into fresh minimal media. *T. atroviride* spores were

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inoculated into this mycelial suspension filtered and concentrated as previously described. Each treatment had three replications and the whole experiment was repeated once.

The effect of physical contact between *T. atroviride* and *S. sclerotiorum* on the expression of NAGase. One 47 mm sterilized 0.45 μm filter was put onto a Petri dish containing 25 mL of minimal media broth. One 0.06 cm^2 plug of *T. atroviride* from potato dextrose agar was inoculated at the center of the filter and two 0.06 cm^2 plugs of *S. sclerotiorum* from potato dextrose agar were inoculated outside the filter. The mycelia of *T. atroviride* and *S. sclerotiorum* were filtered with a 0.45 μm filter just before *T. atroviride* grew off the filter but had not contacted *S. sclerotiorum* mycelia. The other treatment was 3 days after *T. atroviride* and *S. sclerotiorum made* contact. The filtrates were vacuum concentrated as previously described. Each treatment had ten plates and the whole experiment was repeated twice.

The β-N- acetyl-D-glucosaminidase assay. β-Nitrophenyl N-acetyl-β-D-glucosaminide, (Sigma, St. Louis, MO) was used as the substrate for β-N-D-acetyl-glucosaminidase (EC 3.2.1.30) (NAGase) (8). One unit activity was defined as the release of 1 μmol nitrophenol from the substrate per minute (8). The reaction mixture containing 30 μl of enzyme solution and 50 μl of 100 μg/ml substrate was incubated at 50 °C for 15 minutes, then 50 μl of 0.4 M sodium carbonate was added and absorbance was measured at 410 nm.

Determination of protein. Protein concentration was determined by using Bradford Reagent (Sigma, St. Louis, MO) (1). The reaction mixture containing 2 mL of 30 % Bradford Reagent and 50 μL of enzyme preparation was incubated for 30 minutes at

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room temperature before measuring absorbance at 595 nm absorbance and compared with a standard curve using bovine serum albumin.

β-N-acetyl-D-glucosaminidase activity detection with gel electrophoresis. A 12 % Pre Cast Polyacrylamide Electrophoresis Gel (Gradipore™) (Gradipore, Frenchs Forest, Australia) was used to detect β-N- acetyl-D-glucosaminidase (NAGase) activity. The gel was run as described by the manufacturer and then the SDS was removed following a slightly modified procedure from that described (12). Briefly, the gel was washed with cold buffer for 20 minutes three times and then was washed with 50 mM pH 7.0 potassium phosphate buffer for 30 minutes. The washed gel was overlaid with a replica gel (containing 1% agarose, 200 µl/ml of 4-methylumbellifery-N-acetyl-β-D-glucosaminide (MU) in 50 mM, pH 7.0, potassium phosphate buffer). The gel and replica gel were incubated at 50 °C for 1 hour before the replica gel was observed under UV.

The determination of NAGase size. The bands with NAGase activity on the SDS-PAGE were cut out and re-run on SDS-PAGE with a marker to test the activity again as described above and stained with Gradipure®-Electrophoresis stain (Gradipore Ltd, Frenchs Forest, Australia) overnight to determine the molecular weight.

Results

The effect of plant pathogenic fungi on the expression of NAGase. The expression of NAGase was affected by plant pathogenic fungi and only a 93 kDa NAGase was detected when *T. atroviride* was grown with autoclaved mycelia of other fungi (Figure 8.1). The expression of NAGases was different when *T. atroviride* was grown with live cultures of

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B. cinerea, *P. capsici* and *R. solani which* induced *T. atroviride* to produce the 93 kDa NAGase. *S. sclerotiorum* induced *T. atroviride* to produce two NAGases (one was the 93 kDa one and the other was 73 kDa) (Figures 8.1 and 8.6).

The effect of fungal filtrates on the expression of NAGase. *T. atroviride* produced two different sizes NAGase (93 kDa and 73 kDa size), while autoclaved filtrates of *S. sclerotiorum* induced the production of only the 93 kDa NAGase (Figure 8.2). Filtrates of *B. cinerea* induced *T. atroviride* to produce only the 93 kDa NAGase whether it was autoclaved or not (Figure 8.3).

The effect of mycelia on the expression of NAGase. Only the 93 kDa NAGase were produced when ground mycelia or a mycelial extract of were used as the inducer (Figure 8.5).

The effect of physical contact between *T. atroviride* and *S. sclerotiorum* on the expression of NAGase. The expression of NAGase was affected by the physical contact between *T. atroviride* and *S. sclerotiorum*. There were two NAGases produced before the physical contact of *T. atroviride* with *S. sclerotiorum* (Figure 8.5) but only the 73 kDa NAGase was expressed after three days of physical contact between these two fungi.

Discussion

The two bands detected by SDS-PAGE were two different NAGases. Native gel electrophoresis confirmed the differences in these two NAGases (Figure 8.4). This was also confirmed by partial purification of the 93 kDa NAGase from *T. atroviride* and the NAGases extracted from SDS-PAGE (Figure 8.6). The enzyme of 93 kDa NAGase may

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be the same as CHIT 102; however, needs to be purified, sequenced and compared with the CHIT 102 sequence to confirm.

S. sclerotiorum produced NAGase after 10 days growth in minimal media and its size was between the two NAGases expressed by *T. atroviride* (Figure 8.4). The NAGase active bands on SDS-PAGE are not the same NAGase produced by *T. atroviride* because there was no NAGase activity detected from the gel of *S. sclerotiorum* grown in minimal media for 4 days (Figure 8.4). Different plant pathogenic fungi induced different chitinases by several *Trichoderma* species (6,7). The difference also appeared in the *T. atroviride* biocontrol systems. *T. atroviride* produced two different sizes NAGase only when *T. atroviride* was grown with *S. sclerotiorum* or in the filtrates of *S. sclerotiorum* (Figures 8.1 and 8.2). Autoclaved mycelia, non-autoclaved ground mycelia, extractions from mycelia and autoclaved filtrates of *S. sclerotiorum* only induced the 93 kDa NAGase (Figures 8.1, 8.2, 8.4 and 8.5). That means expression of the 73 kDa NAGase was regulated by some extracellular metabolites produced by *S. sclerotiorum*. These extracellular metabolites lost their capacity to induce the expression of 73 kDa NAGase when they were autoclaved. The structure of these extracellular metabolites may be changed during autoclaving and they may not be chitin degradation products such as N-acetylglucosamine (4,12), di-N-acetylchitobiose (12), or tri-N-acetylchitotriose (12) but may be proteins or nucleic acids.

Two NAGases, CHIT 102 and CHIT73, were detected from *T. harzianum strains* T-Y and TM when they parasitized *Sclerotium rolfsii* (6,7). The expression pattern showed that *T. harzianum* produced only CHIT 102 before it came in contact with *S. rolfsii*, and

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CHIT 102 diminished after 12 h and 24 h contact when CHIT 73 was expressed at a high level (7). This interaction was also confirmed by Zeilinger et al. (20) who showed that *nag1* (73 kDa NAGase gene) expression occurred only after *T. harzianum* and *R. solani made* contact (20). The 73 kDa NAGase expressed by *T. atroviride* showed a different pattern since it was contact-independent and triggered by diffusible factor as endochitinase, CHIT 42 (4,12). Also, 73 kDa NAGase activity can be detected before contact is made between *T. atroviride* and *S. sclerotiorum* (Figure 8.5) and when *T. atroviride* is grown in filtrates of *S. sclerotiorum*.

Deoxynivalenol produced by *F. culmorum* and *F. graminearum* suppressed NAGase (11). The fungi tested in this study did not repress NAGase; however, metabolites from *S. sclerotiorum* changed the expression pattern of NAGases. This may be one of the defense reactions of plant pathogenic fungi to *T. atroviride*. Chitinases produced by *Trichoderma* species are complex (6) and the groups and size of chitinases vary with the isolate (10). The regulation of NAGase expression by *T. atroviride* is complex and different from other *Trichoderma* species. Future research is needed to purify and characterize the two NAGases produced by *T. atroviride* in order to determine the in function in biocontrol by *T. atroviride*. Research is also needed to isolate the metabolites produced by *S. sclerotiorum* and show how they regulate the expression of NAGases.

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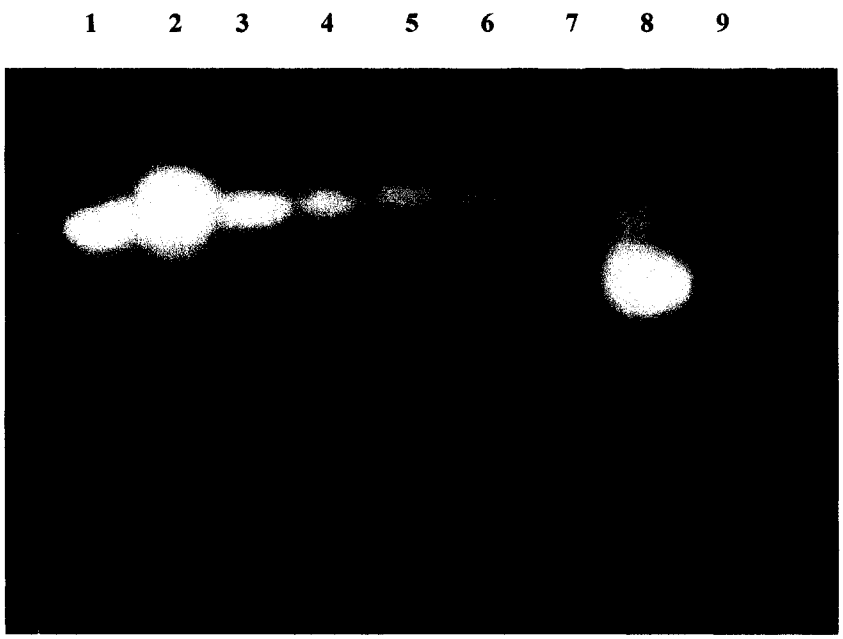

Figure 8.1. NAGase produced by *T. atroviride* with different pathogens. Lanes 1, 3, 5 and 7 were minimal medium supplemented with 0.05 % autoclaved mycelia of *B. cinerea*, *P. capsici*, *R. solani* and *S. sclerotiorum*, respectively; lanes 2, 4, 6 and 8 were combined with the *T. atroviride* and plant pathogens inoculated into minimal medium at the same time; lane 2, *B. cinerea*, lane 4, *P. capsici*, lane 6, *R. solani* and lane 8, *S. sclerotiorum*. Lane 9, minimal medium supplemented with glucose. Each lane was 0.3 μg proteins.

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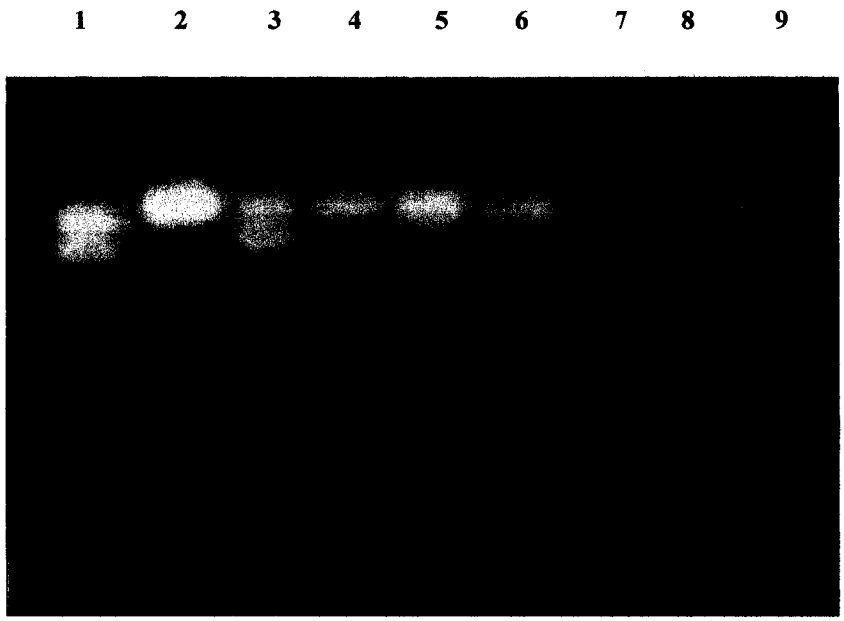

Figure 8.2. The effect of *S. sclerotiorum* filtrate on the expression of NAGase. Lanes 1, 3 and 5, were filtrates from liquid media where *S. sclerotiorum was* grown in minimal media for one day, two days and three days, respectively. Lane 2, lane 4 and lane 6, were autoclaved filtrate from liquid media where *S. sclerotiorum* was grown in minimal media for one, two or three days, respectively; lane 8, *S. sclerotiorum* grown in minimal media for 4 days; lane 9, *S. sclerotiorum* grown in minimal media for 10 days. Each lane was 0.3 μg protein.

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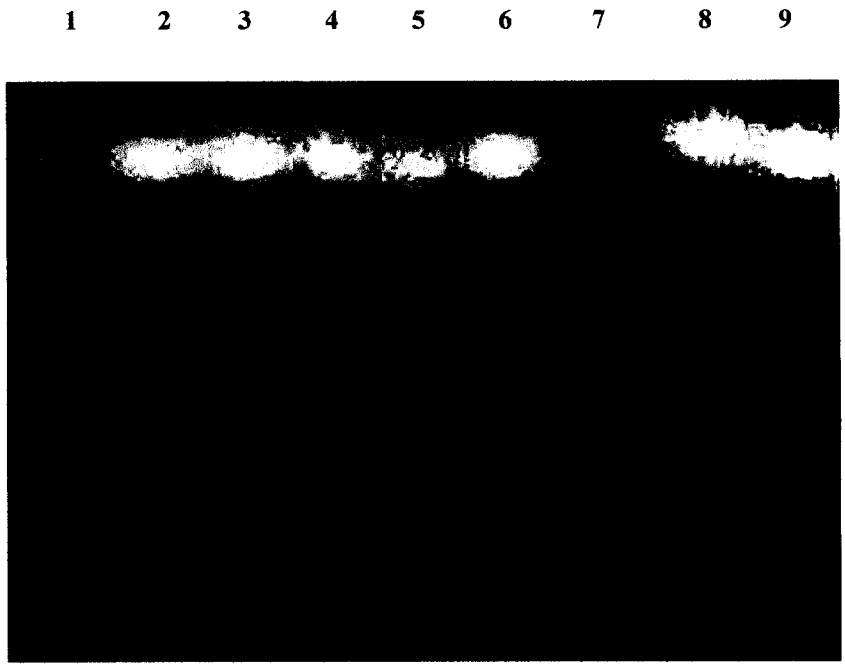

Figure 8.3. The effect of *B. cinerea* filtrate on NAGase expression. Lanes 1, 3 and 5 are where *T. atroviride* grown in filtrates from liquid media for one, two or three days, respectively. Lanes 2, 4 and 6 were where *T. atroviride* was grown in autoclaved filtrates similar to lane 1, 3 and 5. Lane 7, *B. cinerea* grown in minimal media for 4 days. Lane 8, *B. cinerea* and *T. atroviride* grown in minimal media together. Lane 9, *T. atroviride* grown in minimal media supplemented with glucose.

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Figure 8.4. The expression of NAGase in native gel. Lanes 1 and 3 are *T. atroviride* grown in minimal media supplemented with 0.05 % autoclaved mycelia of *B. cinerea* and *S. sclerotiorum*; lanes 2 and 4, are *B. cinerea* and *S. sclerotiorum* inoculated with *T. atroviride* at the same time; and lane 5 was *T. atroviride* grown in minimal media supplemented with 0.05 % glucose. Each lane was 0.3 μg protein.

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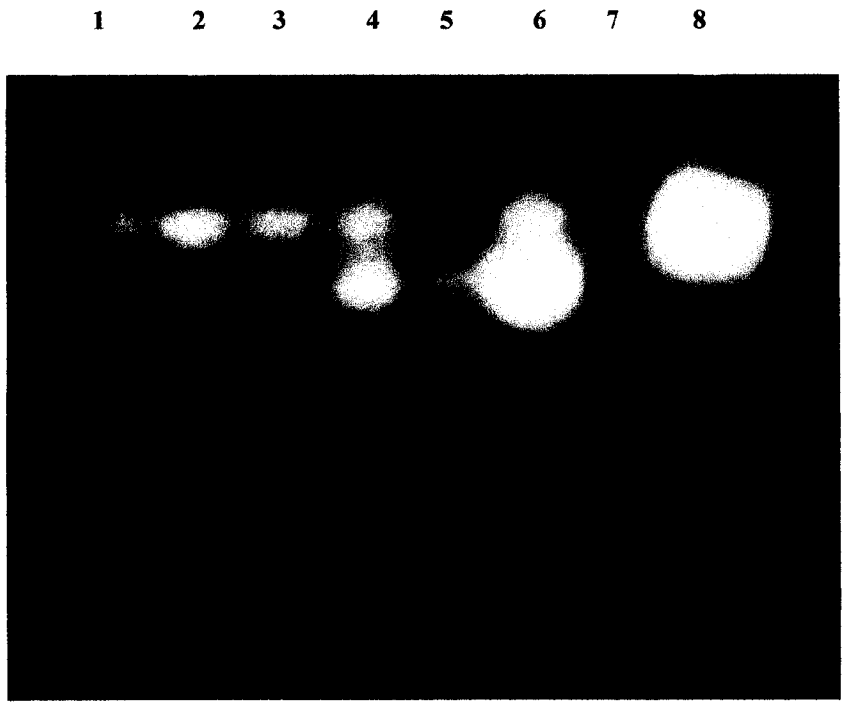

Figure 8.5. The effect of physical contact on the expression of NAGase. Lane 1, from *T. atroviride* grown in minimal media supplemented with 0.05 % glucose. Lane 2, from *T. atroviride* grown with ground 4-day old *S. sclerotiorum* mycelia (not autoclaved). Lane 3, from *T. atroviride* grown with extracted solution from ground 4-day old *S. sclerotiorum* mycelia. Lane 4, before physical contact was made between *T. atroviride* and *S. sclerotiorum*. Lane 5, after 3 days physical contact between *T. atroviride* and *S. sclerotiorum*. Lane 6, *T. atroviride* and *S. sclerotiorum* inoculated into minimal media at the same time. Lane 7, no sample. Lane 8, *S. sclerotiorum* grown in minimal media for 10 days. Lanes 1-5 were 0.3 μg protein. Lanes 6 and lane 8 were 0.5 μg protein.

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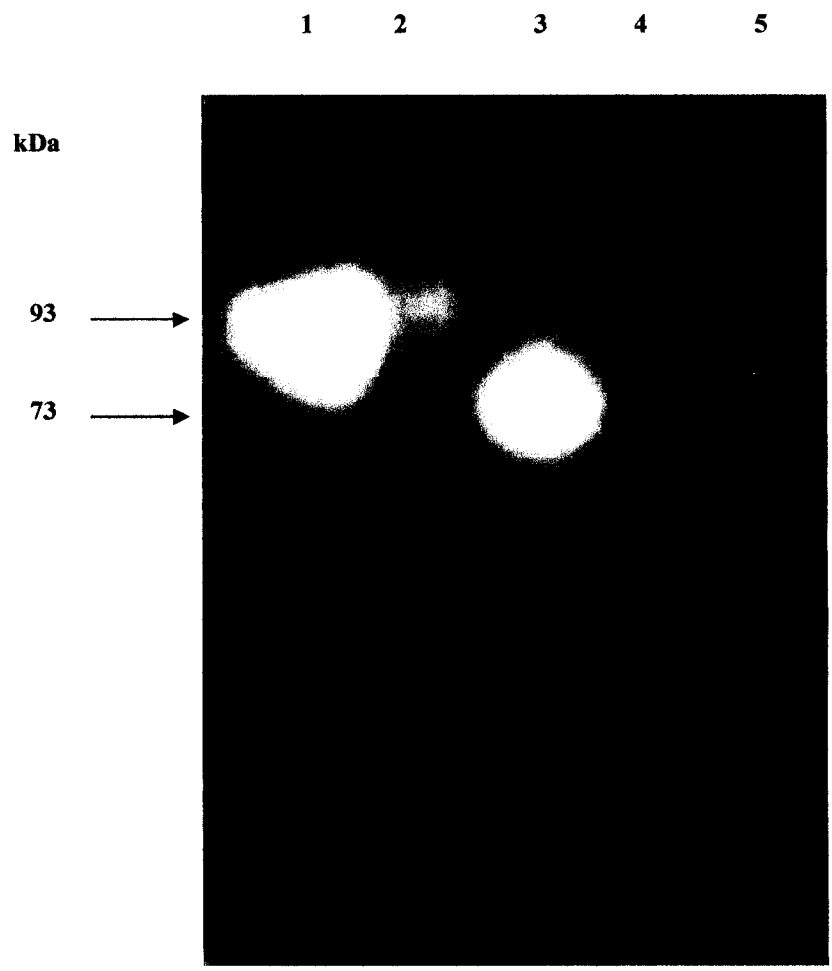

Figure 8.6. Activity of partialy purified NAGases. Lane 1, partialy purified 93 kDa NAGase from chromatofocusing chromatography and gel filtration. Lane 2, the band cut from SDS-PAGE with 93 kDa NAGase activity and re-run on SDS-PAGE. Lanes 3-5, bands cut from SDS-PAGE with 73 kDa NAGase activity and re-run on SDS-PAGE.

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CHAPTER 9: GENERAL CONCLUSIONS

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Enzymatic mechanisms involved in mycoparasitism and biocontrol by *Trichoderma atroviride*

Enzymes produced at room temperature. *Trichoderma atroviride* 901 is a cold tolerant strain isolated from Alaska that can parasitize a wide range of plant pathogens (23). The mechanisms involved in mycoparasitism by *T. atroviride* are similar to other *Trichoderma* species (4). *T. atroviride* produces β-N-acetyl-D-glucosaminidase (NAGase), exochitinase, endochitinase, endo-β-1,3-glucanase, β-1,6-glucanase and proteinase when was grown in minimal media supplemented with autoclaved mycelia of several plant pathogenic fungi (*Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) or glucose at room temperature. The specific enzymes produced were pathogen dependent. The cell wall degrading enzymes induced by autoclaved mycelia of *B. cinerea* peaked within 4-6 days while enzymes induced by other plant pathogens peaked during the first three days. This indicates that different cell wall and cytoplasmic components become available in a sequential manner. Autoclaved mycelia are less complex than cell walls of living plant pathogens because living cells also contain cytoplasmic components. Using autoclaved mycelia of plant pathogenic fungi as a carbon source provides a simpler way to determine the responses of *Trichoderma* with its host. *T. atroviride* also produced several cell wall degrading enzymes when glucose was used as the sole carbon source. The specific activity of cell wall degrading enzymes produced by *T. atroviride* were higher when a low concentration of glucose (0.05 %) was used as the sole carbon source than when autoclaved mycelia of *P. capsici*, *R. solani*, and *S. sclerotiorum* were used. β-1,3-glucanase, β-1,6-glucanase,

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and endochitinase were inhibited by a high concentration of glucose (10 %) while NAGase was less affected by glucose concentration (7). In contrast to *T. harzianum*, exochitinase and endochitinase of *T. atroviride* could be detected only with the high glucose concentration. Some metabolites from autoclaved mycelia of *P. capsici*, *R. solani* and *S. sclerotiorum* may play the same role as deoxynivalenol (DON) from *Fusarium* (17) and repress the expression of cell wall degrading enzymes of *T. atroviride* since the specific activities of cell wall degrading enzymes with these fungal mycelia were lower than with glucose.

Enzymes produced at 7 °C. Temperature is a determining factor for efficacy of biological control of plant diseases. At 7 °C, β-1,4-N-acetyl-D-glucosaminidase (NAGase), exochitinase, endochitinase, β-1,3-endoglucanase and proteinase activity were all higher in the presence of mycelia of fungal pathogens than in its absence (23). The total activity of all chitinases increased over a 30-day period. Increased specific activity of NAGase, exochitinase and endochitinase were detected 5 days after inoculation. Autoclaved mycelia of *B. cinerea, P. capsici, R. solani* and *S. sclerotiorum* induce chitinase (NAGase, exochitinase and endochitinase) and β-1,3-glucanase production. Specific enzyme production of *T. atroviride* was highly dependant on the specific pathogen mycelia used.

Serine proteinase. Proteins are components of the cell wall of pathogenic fungi and proteinases produced by *Trichoderma* may be involved in the parasitism of plant pathogenic fungi (9, 12, 13, 14, 15, 16, 19). *T. atroviride* produces at least two different proteinases. An 18.8 kDa serine proteinase was purified to electrophoretical homogeneity.

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This proteinase may be involved in the biocontrol of phytopathogenic fungi by *T. atroviride* since autoclaved mycelia of plant pathogens can be used as the only carbon source, and the proteinase has strong antifungal activity by inhibiting germination of *B. cinerea* conidia. Cell wall components of fungi vary by species and growth stages (6) which may explain why the 18.8 kDa proteinase was produced after different incubation times with autoclaved pathogen mycelia. The first 10 N-terminal amino acids of the reported 18.8 kDa proteinase are IVGGTTAAAG. It is similar to a 27 kDa trypsin proteinase from *T. harzianum* (19) and a 25 kDa serine trypsin-like proteinase from *T. viride* (22), but different from the 18.8 kDa proteinase of *T. harzianum* (9) and a serine proteinase from *T. virens* (16). The reported 18.8 kDa proteinase is a new by characterized proteinase associated with biocontrol by *T. atroviride*. The 18.8 kDa proteinase produced by *T. atroviride* was inhibited by 0.05 % glucose and no proteinase bands appeared when glucose was added to the minimal media. That indicated glucose may inhibit 18.8 kDa serine proteinase production.

β-1,3-glucanase. At least two β-1,3-glucanase bands were detected from *T. atroviride* 901 by SDS-PAGE, and three β-1,3-glucanase bands appeared on IEF gel. A 77.8 kDa endo-β-1,3-glucanase was purified from cold tolerant *T. atroviride* 901 that showed strong antifungal activity by inhibiting spore germination of *Botrytis cinerea*. The purified 77.8 kDa enzyme was classified as an endo-β-1,3-glucanase by its inability to hydrolyze ρ-Nitrophenyl-β-D-glucopyranoside (2, 21). The activity of endo-β-1,3-glucanase at 6 °C was 0.29 U, or about 22 % of its activity at 45 °C. Activity of endo-β-1,3-glucanase at 15 °C was about 77 % of its activity at 45 °C. These results confirm that

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cold-tolerant *T. atroviride* 901 has antifungal activity and produces the extracellular ability to control pathogenic fungi at low temperature (3°C-10°C) (23). The first ten N-terminal amino acid sequence of purified endo-β-1,3-glucanase was similar to a α-1,3-glucanase associated with biocontrol by *T. harzianum* with only one amino acid (the tenth) difference (1). However, substrates specificity, the molecular weight, pI value and Km of the purified endo-β-1,3-glucanase from *T. atroviride* was different from other *Trichoderma* endo-β-1,3-glucanases (8,20). The optimal pH for the *T. atroviride* endo-β-1,3-glucanase was 4.0, the optimal temperature was 45 °C and the inactivation temperature was 50 °C.

The 73 kDa β -N-acetyl-D-glucosaminidase genes. The three 73 kDa β -N-acetyl-D-glucosaminidase genes were amplified from cold tolerant *T. atroviride* biotypes 861, 603, and 453 by Polymerase Chain Reaction (PCR). The PCR product was about 2.7 kb. Sequences of the genes for strains 861, 603, 453, and conducted by forward and reverse orientations produced 2416, 2418 and 2564 base pairs, respectively. Eleven unique base pairs from *T. atroviride* biotypes were identified by comparing them with the published β -N-acetylglucosaminidase gene from GeneBank that showed the number of base pair insertions was 4, 5 and 5, and single base pair change was 13, 13, and 9 for 603, 453 and 861, respectively. The genes of these three cold tolerant *T. atroviride* biotypes are different from the published gene of *T. harzianum.* Similarity of mutants 603 and 453 were high and they were distinct from wild type 861. Single and multiple nucleotide polymorphisms may be used to determine the phylogenetic similarities of different *Trichoderma* species and strains.

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β-1,6-glucanase. β-1,6-glucan is a minor part of plant pathogens cell walls, but β-1,6-glucanase may be involved in biocontrol by *Trichoderma*. β-1,6-glucanases produced by cold tolerant *T. atroviride* were influenced by carbon sources and pH, and multiple β-1,6-glucanases were produced when it was grown with different carbon sources. *T. atroviride* produced β-1,6-glucanase when it was grown with glucose, glycol chitosan, pustulan and autoclaved mycelia of plant pathogens. The expression of different β-1,6-glucanase isozymes with the various carbon sources indicates that the carbon structure and cell wall component differences were important factors (3, 6). At least three different molecular weight β-1,6-glucanase isozymes were detected from *T. atroviride* under inducing conditions. Starvation may be needed for β-1,6-glucanase production similar to endochitinase (CHIT42) and N-acetyl-D-glucosaminidase (NAG1) (10) since yeast (high percentage of β-1,6-glucan in the cell walls) cannot induce *T. atroviride* to produce β-1,6-glucanase. Further studies to characterize the two new β-1,6-glucanase isozymes of *T. atroviride* are needed before their role in biocontrol is clear.

The effect of plant pathogens in the expression of NAGase. β-N-acetyl-D-glucosaminidase is one of the important enzymes involved in *T. atroviride* biocontrol. Two β-N-acetyl-D-glucosaminidases were produced by *T. atroviride* when it was grown with plant pathogenic fungi (*Botrytis cinerea, Phytophthora capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum*) or with autoclaved mycelia as verified by SDS-PAGE and native PAGE. One is about 93 kDa and the other is about 73 kDa. The 73 kDa β -N-acetyl-D-glucosaminidase was produced only when *T. atroviride* was grown with mycelia of *S. sclerotiorum*. Culture filtrates of *S. sclerotiorum* regulated production of

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the 73 kDa β-N-acetyl-D-glucosaminidase which culture filtrates of *B. cinerea* had no effect its expression. Since expression of the 73 kDa β -N-acetyl-D-glucosaminidase occurred before physical contact of *T. atroviride* with the *S. sclerotiorum* mycelia occurred, NAGase expression was regulated by some soluble component from *S. sclerotiorum*. These extracellular metabolites lost their capacity to regulate the expression of 73 kDa NAGase when the filtrates were autoclaved, and may not be chitin degradation products such as N-acetylglucosamine (5, 18), di-N-acetylchitobiose (18), or tri-N-acetylchitotriose (18), but may be proteins or nucleic acids.

Expression of the 73 kDa NAGase produced by *T. atroviride* was contact-independent and triggered by a diffusible factor similar to endochitinase CHIT 42 (5, 18). This was shown by detection of 73 kDa NAGase activity before contact between *T. atroviride* and *S. sclerotiorum*, and by cell-free filtrates of *S. sclerotiorum*. This change in expression pattern of NAGases may be one of the defense reactions of plant pathogenic fungi to *T. atroviride* (11).

Future research. The *T. atroviride* biocontrol system is more complex than we initially thought. There are still some enzymes that need to be purified and characterized in order to determine the function of these enzymes in biocontrol by *T. atroviride*. Enzymes produced by *T. atroviride* include at least one proteinase, one or two β-1,3-glucanases, two β-1,6-glucanases and two NAGases.

Future research also should isolate the metabolites produced by *S. sclerotiorum* and show how they regulate the expression of NAGases. Another important future work is to characterize the enzymes and proteins of *T. atroviride* grown at low temperature.

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